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— SECTION E —

# MEDICAL SCIENCES

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# THE EFFECT OF ACUTE STARVATION ON THE BODY ORGANS OF THE ADULT WHITE RAT, WITH ESPECIAL REFERENCE TO THE ADRENAL GLANDS[1]

BY A. T. CAMERON[2] AND J. CARMICHAEL[3]

## Abstract

When rats are starved by withholding all food, though giving water, they develop, two or three days before death, a typical behaviour syndrome, in which at first there is increased activity, and subsequently, in old animals, marked immobility. At this pre-mortal stage in old animals, or the corresponding stage in younger rats, they have lost 30% or more of their body weight, and if they are killed the adrenals are found to be enlarged and discoloured to a dark grey or dark dirty grey. These enlarged glands contain an increased amount of water though their solid content remains practically unaltered, the change representing an increase in the water content of the cells (possibly confined to the cortex). The glomerular zone is diminished in thickness, and the reticular zone also shows abnormality. Animals killed before they have lost 30% of their body weight still have normal adrenals (normal in size, weight, water content, and colour). *The enlargement produced in the pre-mortal stage of inanition is a pathological process and is not a hypertrophy.* Data in the literature suggest that certain types of adrenal enlargement such as that produced by deficiency of the B-complex vitamins may be similar in nature. The kidneys, heart, and gonads of acutely starved rats tend to lose weight more slowly than the whole body; the liver and spleen more rapidly; and the thyroid at about the same rate.

## Introduction

This paper embodies the results of an investigation that developed from a comparison of the relative effects of the feeding of oestrogens and of chronic starvation, whose results will be published separately. In agreement with previous reports (14, 22) we were unable to demonstrate enlargement of the adrenals in chronic starvation (5) so that the acute starvation experiments were directed particularly to determining the occurrence or otherwise of such enlargement.

Jackson (15) in 1919 found some evidence of degeneration of the fascicular zone of the adrenal cortex and of the medulla in acutely starved rats. He made no mention of enlargement. Enlargement of the adrenal glands in pigeons as result of starvation was reported by McCarrison (16) and confirmed by Vincent and Hollenberg (29) who also found it to occur in dogs and rats. It has been attributed to deprivation of B-complex vitamins during starvation (3), while the enlargement produced by a diet deficient in these vitamins has

[1] *Manuscript received January 3, 1946.*

*Contribution from the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Man.*

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[3] *Technician.*

been attributed to resulting loss of appetite and consequent partial starvation (6), although, as mentioned above, chronic starvation does not produce adrenal enlargement.

When the earlier reports of such enlargement were made (16, 29) the function of the adrenal cortex was not known, and adrenal function was thought of in terms of adrenine content. Evidence was adduced that the adrenine content of such enlarged glands was greater than normal, so that both McCarrison and Vincent considered the enlargement to be a hypertrophy. Later evidence concerning the adrenine content of such glands is conflicting (3, 19, 20, 27, 30).

In 1943, Tepperman, Engel, and Long (27), in a critical and well-documented review of the literature dealing with hypertrophy of the adrenal cortex, found that most investigators agreed that complete inanition produces adrenal hypertrophy. Goldzieher (11) in a still later review holds the same view.

In most acute starvation experiments water has not been withheld.

## Experimental Part

In all experiments an equal number of control rats were kept in the same environmental conditions, and fed the usual diet of unlimited bread and milk and table scraps during the experimental period. The starved animals were given unlimited water. All animals were isolated in separate cages.

The earlier experiments were carried out with fairly old female rats. (In an initial measurement of the survival period, that of six animals varied from six to nine days; they lost from 31 to 39% of body weight before death.) It was found that animals killed after five days' starvation seldom showed adrenal enlargement, and the period was gradually extended. Careful observation indicated a fairly regular pattern of behaviour in these elderly animals. During the first few days nothing unusual was observable. About 48 hr. before death would occur the starved rats became more active than the controls, and the increased activity persisted at least 24 hr. and sometimes 36 hr. and more. Subsequently the animals became inactive and were usually curled up in a ball, scarcely responding at all to such a stimulus as tapping the cage. At this period they had always lost at least 30% of body weight. Those animals allowed to live longer died during the following night or were moribund next morning. If they were killed (by chloroforming) during the day on which the immobility was first observed or subsequently, before actual death from starvation took place, the adrenals were almost always found to be definitely enlarged, and of a dark grey (or dark dirty grey) colour. Such animals did not struggle while going under the anaesthetic, but breathed slowly and deeply for a short while and then suddenly keeled over.

Old male rats behaved similarly. In younger animals the onset of immobility was less noticeable or did not occur. In the late stage with these younger animals, when they had lost well over 30% of their weight, the eyes remained partially closed, and the stance of the animals was continuously "compressed". Killed at this stage these animals also almost always showed enlarged and discoloured adrenals.

In all, 12 experiments were carried out with 70 rats, half of which were starved. Of the latter, three died during the night and were not examined, two were examined just after death had occurred, and the remainder were killed with chloroform. The controls were killed at about the same time. The adrenals in eight controls and seven starved animals, and the thyroids in five controls and five starved animals were examined histologically. The method of dissection, etc., employed has been described elsewhere (4).

The general results are most easily recorded by separating the starved animals into two groups, (i) animals in which the loss of body weight was less than 30%, and (ii) those in which it was 30% or more. Variations in the organ weights of different animals, and indeed in the individual percentage organ weights, were considerable, both in controls and starved animals, and averages probably give a sufficiently accurate idea of the general results.

In the older rats the change in weight of the controls during the brief experimental period was so small (usually within $\pm 2\%$) that their final weights could properly be used to calculate the percentage organ weights, but the initial body weights of the starved animals had to be used. In younger and still growing animals, conclusions were drawn by contrasting percentage organ weights based on initial with those based on final body weights in the manner described later.

*Rate of Loss of Body Weight*

For 17 fairly old females (initially 186 to 259 gm.) the rate of loss of body weight averaged for the first few days about 8 gm. daily (extremes 4 to 10 gm.). There was no definite relation between initial weight and weight loss. For six old males (178 to 322 gm.) the average loss was about 10 gm. (extremes 8 to 12 gm.). For seven younger males (112 to 134 gm.) the average daily loss was about 7 gm. (extremes 6.5 to 8 gm.). There was a general tendency to a slightly more rapid fall of weight in the last day or two.

*Loss of Weight of Certain Body Organs in Older Animals*

The starved animals showed general loss of body fat. The amount was not determined. The average results for the body organs examined are shown in Table I.

TABLE I

GAIN OR LOSS OF CERTAIN BODY ORGANS OF OLD RATS PRODUCED BY STARVATION

| Sex | No. | Original body wt., gm. | | Loss of body wt., % | | Average loss or gain of organs, % | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Extremes | Mean | Extremes | Mean | *L* | *K* | *H* | *S* | *M* | *G* | *A* | *T* |
| Female | 9 | 192 to 257 | 214 | −16 to −29 | −22.5 | −30 | −16 | −13 | −23 | −22 | − 5 | − 2 | − 9 |
| | 9 | 186 to 259 | 213 | −31 to −45 | −36 | −50 | −24 | −23 | −52 | −30 | −16 | +29 | −30 |
| Male | 2 | 224 to 322 | 273 | −24 to −26 | −25 | −38 | −27 | −21 | −19 | −14 | − 6 | − 7 | +30* |
| | 3 | 226 to 314 | 279 | −30 to −34 | −32 | −68 | −33 | −30 | −53 | −23 | −18 | +20 | −31 |

* *One thyroid was greatly enlarged for some unascertained reason. Its water content was normal.*

*L, liver; K, kidneys; H, heart; S, spleen; M, right anterior tibialis muscle; G, gonads (ovaries or testes); A, adrenals; T, thyroid.*

The results with female rats indicate that during the period when weight loss is less than 30% the liver loses weight relatively more rapidly than the whole body; the spleen and muscle at about the same rate; kidneys, heart, thyroid, and ovaries more slowly; while the adrenals are practically unaffected. With greater loss of body weight the spleen also loses weight relatively faster than the whole body, the others relatively less rapidly, while the adrenals gain weight.

The number of male animals was too small to stress the results obtained with them, but the general picture is the same.

TABLE II

EFFECT OF STARVATION WEIGHTS ON WEIGHTS OF BODY ORGANS OF YOUNG ADULT MALE RATS

| | Rat 1 (control) | Rat 2 (control) | Rat 3 (starved) | Rat 4 (starved) |
|---|---|---|---|---|
| Body weight, gm. | | | | |
| Initial | 111 | 114 | 114.5 | 112 |
| After 5.3 days | — | — | — | 69.5 |
| After 6.0 days | 137 | 135.5 | — | — |
| After 6.3 days | — | — | 69.5 | — |
| Gain or loss | +26 | +21.5 | −45 (−39%) | −42.5 (−38%) |
| Organ weights, gm. | | | | |
| Liver | 8.7 | 9.6 | 3.4 | 2.0 |
| Kidneys | 1.62 | 1.52 | 0.98 | 0.84 |
| Heart | 0.47 | 0.47 | 0.29 | 0.30 |
| Spleen | 0.48 | 0.45 | 0.11 | 0.11 |
| Muscle* | 0.26 | 0.24 | 0.17 | 0.14 |
| Testes | 1.82 | 1.56 | 1.33 | 1.35 |
| Adrenals | 0.0280 | 0.0302 | 0.0355 | 0.0365 |
| Thyroid | 0.0135 | 0.0149 | 0.0074 | 0.0070 |
| Dry weights: adrenals | 0.0088 (31%) | 0.0087 (29%) | 0.0091 (26%) | 0.0087 (24%) |
| Organ weights referred to orig. body wt., % | | | | |
| Liver | 7.8 | 8.4 | 2.1 | 1.8 |
| Kidneys | 1.46 | 1.33 | 0.86 | 0.74 |
| Heart | 0.42 | 0.41 | 0.25 | 0.26 |
| Spleen | 0.40 | 0.40 | 0.09 | 0.09 |
| Muscle* | 0.23 | 0.21 | 0.15 | 0.12 |
| Testes | 1.64 | 1.37 | 1.16 | 1.19 |
| Adrenals | 0.0252 | 0.0265 | 0.0310 | 0.0320 |
| Thyroid | 0.0121 | 0.0131 | 0.0065 | 0.0061 |
| Organ weights referred to final body wt., % | | | | |
| Liver | 6.4 | 7.1 | 4.8 | 2.9 |
| Kidneys | 1.18 | 1.11 | 1.27 | 1.21 |
| Heart | 0.34 | 0.34 | 0.41 | 0.42 |
| Spleen | 0.35 | 0.34 | 0.16 | 0.15 |
| Muscle* | 0.19 | 0.18 | 0.25 | 0.20 |
| Testes | 1.33 | 1.15 | 1.91 | 1.94 |
| Adrenals | 0.0204 | 0.0223 | 0.0511 | 0.0532 |
| Thyroid | 0.0098 | 0.0110 | 0.0106 | 0.0101 |

* *Right tibialis anterior muscle.*

The conservation of weight of the gonads seemed to be associated with conservation of function. About half of the relatively old female rats (both starved and control animals) showed numerous haemorrhagic follicles.

*Results with Younger Animals*

The protocol of a typical experiment with young rats is given in Table II. Four males of the same litter, born Dec. 2, 1944, were used, and the experiment was started on the 53rd day of age.

It seems proper to analyse results such as those in Table II in the following way:

| | Compared with controls | Conclusion |
|---|---|---|
| Actual organ weights | Greater | Actual enlargement |
| Percentage organ weights referred to initial body weight | Greater | Actual enlargement |
| Percentage organ weights referred to initial body weight | Equal | No effect |
| Percentage organ weights referred to initial body weight | Less | Relatively less loss than that of whole body |
| Percentage organ weights referred to final body weight | Greater | Relatively less loss than that of whole body |
| Percentage organ weights referred to final body weight | Equal | Loss proportional to that of whole body |
| Percentage organ weights referred to final body weight | Less | Loss relatively greater than that of whole body (with possible actual atrophy) |

Applying such analysis to the results in Table II, they indicate (*a*) atrophy of liver and spleen, (*b*) loss of weight of kidneys, heart, muscle, and testes less in degree than that of the whole body, (*c*) loss of weight of the thyroid proportional to that of the whole body, and (*d*) actual enlargement of the adrenals.

Other experiments with young adult male rats, in which body weight loss exceeded 30%, were in complete agreement. It will be seen that the results are in general agreement with those obtained with older animals of both sexes.

*Effect of Starvation on the Adrenal Glands*

The abnormal appearance of the adrenal glands of animals whose loss of body weight was 30% or more led to determination of their water content. This was done in all cases except when glands were required for histological examination. They were dried for 24 hr. at 102° to 105° C. Results are shown in Table III for, in order, (i) old females, (ii) old males, (iii) younger males. Data for controls precede those for starved animals. The latter are arranged in order of decreasing water content of the adrenals. Presence or absence of enlargement of the adrenals was judged by direct comparison with the adrenals of corresponding controls, and also comparison based on initial body weights. Some of the exceptions to general findings may be due to individual variations.

It is evident that when there is a loss of body weight of 30% or more, in almost every case the adrenals become enlarged, and at the same time dis-

TABLE III

EFFECT OF STARVATION ON THE ADRENAL GLANDS

| Sex | No. of animals | Treatment | Duration, days | Initial body wt., gm. | Gain or loss in body wt., % | Adrenals | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Fresh wt., gm. | Dry wt., % | Colour | Size |
| Female | 11 | Control | (5 to 11) | 176 to 230 | −1 to +3 | 0.0467 to 0.0606 | 30 to 33 | Normal | Normal |
| | 1 | " | (8) | 217 | +1 | 0.0954 | 27 | Dark grey | Large |
| | 1 | " | (11) | 226 | −1 | 0.0847 | 30 | Greyish | Large |
| | 1 | " | (8) | 214 | +2 | 0.0746 | 32 | Grey-yellow | Large |
| Female | 1 | Starved | 11 | 245 | −45 | 0.1762 | 16 | Dark dirty grey | Enlarged* |
| | 1 | " | 11 | 259 | −39 | 0.1012 | 21 | " | " |
| | 1 | " | 9 | 205 | −40 | 0.0586 | 22 | " | ? |
| | 1 | " | 5 | 189 | −32 | 0.0692 | 23 | Dark grey | Enlarged** |
| | 1 | " | 7 | 202 | −32 | 0.0649 | 23 | " | " |
| | 1 | " | 7 | 186 | −36 | 0.0569 | 23 | " | " |
| | 1 | " | 8 | 211 | −31 | 0.0802 | 27 | Almost normal | " |
| | 7 | " | 5 to 8 | 192 to 257 | −29 to −16 | 0.0488 to 0.0637 | 27.5 to 36 | Normal | Normal |
| | 1 | " | 5 | 217 | −18 | 0.0665 | 33 | Normal | Enlarged |
| Male | 5 | Control | (7 to 9) | 224 to 325 | −1 to +2½ | 0.0268 to 0.0376 | 29 to 33 | Normal | Normal |
| | 1 | " | (9) | 311 | −1 | 0.0497 | 26 | Bright yellow | Large |
| Male | 3 | Starved | 6 to 9 | 226 to 296 | −30 to −34 | 0.0359 to 0.0459 | 23 to 23 | Dark grey | Enlarged |
| | 2 | " | (7, 9) | 224, 322 | −24, −25 | 0.0277, 0.0355 | 28.3, 29 | Normal | Normal |
| Male | 5 | Control | (6 to 7) | 97 to 137 | +12 to +23 | 0.0199 to 0.0322 | 29 to 37 | Normal | Normal |
| Male | 1 | Starved | 5.2 | 112 | −38 | 0.0365 | 24 | Dark grey | Enlarged |
| | 1 | " | <6 | 134 | −36 | 0.0337 | 25 | (Dark red)*** | Enlarged |
| | 1 | " | 6.3 | 131 | −34 | 0.0257 | 25 | Dark grey | Normal |
| | 1 | " | 6.2 | 114 | −39 | 0.0355 | 26 | Dark dirty grey | Enlarged |
| | 1 | " | 6.3 | 129 | −31 | 0.0278 | 28 | " | Normal |

* *Adrenals swollen like tiny balloons. When punctured after transfer to weighing vessel, a clear fluid exuded.*

** *When adrenals were punctured after transfer to weighing vessel, a blood-stained fluid exuded.*

*** *Animal died during night but was still warm and not in rigor when examined. The dark red colour of the adrenals was presumably a post-mortem change.*

coloured to a dark grey or dark dirty grey (often like dark mud), while their water content increases. (Occasionally such enlarged glands showed one or two apparently haemorrhagic spots on their surfaces.)

That the content of solid of these enlarged glands did not appreciably alter is shown in Table IV. (Final body weights were used in calculating the ratio of adrenal dry weight to total body weight for the older controls, and initial body weights for all starved animals and for the younger controls.)

*Histological Examination*

Sections of adrenals and thyroids were preserved in 10% commercial formol, or in Bouin's solution, and stained with haematoxylin-eosin. In preliminary experiments no striking differences were observed in the adrenals of starved

TABLE IV

EFFECT OF STARVATION ON THE SOLID CONTENT OF THE ADRENALS

| Group | No. of rats | Adrenal water content, % | Adrenal solid content, % | Ratio of adrenal dry weight to body weight | |
|---|---|---|---|---|---|
| | | | | Extremes | Mean |
| Control females (old) | 14 | 67 to 73 | 33 to 27 | 0.0060 to 0.0118 | 0.0088 |
| Starved females (old) | 8 | 64 to 72½ | 36 to 27½ | 0.0072 to 0.0111 | 0.0084 |
| Starved females (old) | 7 | 73 to 84 | 27 to 16 | 0.0063 to 0.0118 | 0.0085 |
| Control males (old) | 6 | 67 to 74 | 33 to 26 | 0.0033 to 0.0043 | 0.0037 |
| Starved males (old) | 2 | 71 to 72 | 29 to 28 | 0.0031 to 0.0036 | 0.0033 |
| Starved males (old) | 3 | 77 | 23 | 0.0030 to 0.0037 | 0.0034 |
| Control males (young) | 5 | 63 to 71 | 37 to 29 | 0.0061 to 0.0087 | 0.0073 |
| Starved males (young) | 5 | 72 to 76 | 28 to 24 | 0.0048 to 0.0098 | 0.0073 |

and control animals. The cells lining the thyroid alveoli of starved animals tended to be flatter, and the alveolar colloid to stain less.

Since chemical evidence indicated a definite increase in water content in almost all the cases of adrenal enlargement, the only possible conclusion seemed to be that the average cell size was greater. It was thought that a count of nuclei in comparable areas might test this conclusion. In two preliminary experiments, using the method of counting described below, the nuclei in comparable areas were respectively 4 and 13% less in the adrenals of starved rats than in the controls. A third, more thorough experiment was then performed, and is described fully.

Six male rats of the same litter were used. Starvation of three was begun on the 48th day of age; the others served as controls. One of the starved animals died on the sixth day, during a week-end. The pertinent data for the others are given in Table V.

The adrenals were preserved in Bouin's solution (water, 30 parts; formalin, 10 parts; 2% aqueous trichloracetic acid solution, 2 parts; and picric acid to saturation), sectioned, and stained with haematoxylin–eosin. All sections were 7$\mu$ thick. As far as possible, sections were cut at right angles to the longest adrenal axes and to their lower (flatter) sides adjacent to the kidneys. Photomicrographs were made from sections from the central portions of the glands.

Except where flaws in the sections interfered, attempts were made to obtain four photomicrographs from the four opposite points of the peripheries of a section, selecting, where possible, areas with fairly flat peripheries.

In all, 23 photomicrographs were made of the four adrenals of the starved animals, of which 20 (5 × 4) were made from five sections; 29 were made from the six adrenals of the controls, of which 20 (5 × 4) were from five sections.

Where possible, square 10 × 10 cm. areas were used to count nuclei, each area bordering on the peripheral connective tissue but just excluding it. In

TABLE V

DATA CONCERNING RATS USED IN THIRD HISTOLOGICAL EXPERIMENT

| | Rat 1 (starved) | Rat 2 (starved) | Rat 4 (control) | Rat 5 (control) | Rat 6 (control) |
|---|---|---|---|---|---|
| Weight, gm. | | | | | |
| Initial | 126.5 | 127 | 121 | 122.5 | 124.5 |
| After 7.3 days | 75.5 | 72.5 | — | — | — |
| After 8 days | — | — | 146.5 | 148.5 | 150 |
| Gain or loss in weight | −51 (−40%) | −54.5 (−43%) | +25.5 | +26 | +25.5 |
| Right adrenal gland | 0.0166 | 0.0186 | 0.0131 | 0.0150 | 0.0150 |
| Left adrenal gland | 0.0190 | 0.0180 | 0.0127 | 0.0169 | 0.0147 |
| Total adrenal weight | 0.0356 | 0.0366 | 0.0258 | 0.0319 | 0.0297 |
| Average (normals) | | | | (0.0291) | |
| Approximate weight increase (starved rats) | +22% | +26% | | | |
| Adrenal colour | Dark dirty grey | Dark dirty grey | Slightly greyish yellow | Slightly greyish yellow | Dull yellow |

each square, the nuclei were counted in alternate strips, each 1 × 10 cm., running inwards from the periphery. When the edge was markedly curved, five consecutive strips in a total area of 5 × 10 cm. were counted. All such strips included the glomerular and fascicular zones and a portion of the reticular zone.

Counts in 10 consecutive strips and in five alternate strips gave an average difference (11 comparisons) of 0.35% and a maximum difference of 1.7%. Eleven similar comparisons between 10 and 5 consecutive strips gave an average difference of 1.5% (maximum 3.3%).

All "spots" that could possibly be nuclei were counted. Hence the counts were undoubtedly inaccurate and too great, but it seems reasonable to assume that the errors for starved and for control animals were of similar magnitude.

The results showed a random variation with respect to the position of the area counted (adjacent or opposite to the base of the adrenal, etc.). Moreover, counts made in several instances from more than one section of the same gland showed marked differences, although such sections were fairly close to each other (the actual distance apart was not determined). Thus typical actual figures were, per square cm.:

Rat 2 (starved), right adrenal,—

Section A, four photos: extremes 28.2–30.8; mean 29.4.

Section B, four photos: extremes 19.4–22.7; mean 21.0.

Rat 4 (control), left adrenal,—

Section A, three photos: extremes 40.5–47.3; mean 44.9.

Section A, four photos: extremes 35.0–37.8; mean 36.2.

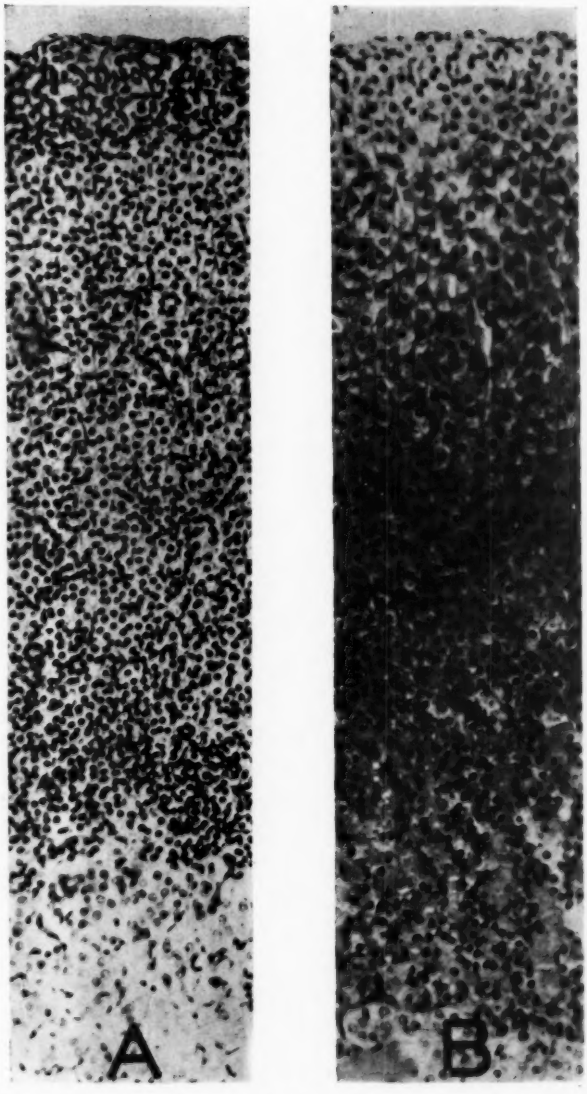


FIG. 1. *Photomicrographs of adrenal sections. ×200. A, left adrenal, Rat 4 (control). In an area 10 × 10 cm., bordering on the periphery, a nuclear count showed 47.3 nuclei per square cm. B, right adrenal, Rat 2 (starved). The corresponding count was only 22.6 nuclei per square cm.*

Hence it seemed useful to compare only the over-all averages, and the averages for the total counts of each adrenal. These (per square cm.) were as follows:

23 photos of sections of 4 adrenals of 2 starved rats averaged 30.2
29 photos of sections of 6 adrenals of 3 control rats averaged 36.4

*Decrease in starved animals, 17.0%.*

7 photos of left adrenal of starved rat No. 1 averaged 32.1
4 photos of right adrenal of starved rat No. 1 averaged 32.8
4 photos of left adrenal of starved rat No. 2 averaged 34.3
8 photos of right adrenal of starved rat No. 2 averaged 25.2

Mean, 31.1.

7 photos of left adrenal of control rat No. 4 averaged 39.9
4 photos of right adrenal of control rat No. 4 averaged 43.5
4 photos of left adrenal of control rat No. 5 averaged 31.4
6 photos of right adrenal of control rat No. 5 averaged 34.0
4 photos of left adrenal of control rat No. 6 averaged 35.6
4 photos of right adrenal of control rat No. 6 averaged 32.7

Mean, 36.2.

*Decrease in starved animals, 14.1%.*

On a volume basis the last figure indicates a difference of about 25%, while the average enlargement in the starved animals was about 24%. This close approximation can only be accidental, since only a small part of each gland was examined, and the medullas not at all.

As is to be expected counts of areas in individual photos of the two groups showed considerable overlapping. The extremes were, for starved animals, 19.4 and 37.0 per square cm., and for controls 29.3 and 47.8.

The average results are in agreement with the supposition that the increase in water content (and lack of increase in solid content) of adrenals of starved rats is accounted for by enlargement of the cells.

Fig. 1 shows photomicrographs of two extreme cases. These indicate that there is a considerable narrowing of the glomerular zone in the starved rats; such decrease was generally present in some degree, as indicated both by inspection of the series of photographs and camera lucida drawings of actual sections. The reticular zone also tends to be abnormal, with frequent spaces empty of nuclei, and presumably indicating a degenerative process. Fig. 1 also suggests that a large proportion of the nuclei in the adrenals of the starved animals are themselves enlarged, sharing in the increased water content.

Study of camera lucida drawings of the sections suggested that the medullas were not enlarged; there were, however, considerable variations of the areas in different sections, and this conclusion cannot be stressed.

## Discussion of Results

The effect of starvation on the body organs of the white rat (excluding the adrenals), as reported in this paper, is in fairly good agreement with the results of Jackson (14) and of Mulinos and Pomerantz (21). The latter investigators withheld water as well as food, and apparently this added deprivation does not materially affect the results.

Vincent and Hollenberg (30) claimed that inanition produces enlargement of the thyroid in the rat. (Some of their published values are at least 10 times too great, apparently through misplacement of a decimal point in printing.) No later observer seems to have confirmed this claim, nor do our findings support it.

The increase in activity for two or three days prior to death in the acutely starved rat accords with observations of Hoskins (13) and of Hitchcock (12).

The fairly constant pattern of behaviour, associated with a fairly definite degree of weight loss, enables animals to be killed at a time when in almost all cases the adrenals are definitely enlarged. To ensure this, the rats must have been unduly active for at least 24 hr., old animals must have reached the subsequent quiescent stage, and body weights must have decreased by 30% or more.

Rats killed at any period when weight loss is less than 30% have normal adrenals.

These findings are in agreement with, and are somewhat more precise than those of Mulinos and Pomerantz for rats (22), and Oleson and Bloor (24) for guinea pigs. The former stated: "At the end of the first week of starvation the adrenal glands are usually heavier, although they may be normal and at times reduced in weight. As the period of starvation lengthens and the body weight loss becomes more severe, the adrenal glands increase in weight. When the starvation was allowed to continue until the rats became moribund, the adrenal glands were invariably enlarged." However, they considered that the enlargement represented true physiological growth (23). Oleson and Bloor reported that the weights of guinea pig adrenals are not changed by a seven day fast, although they are increased in animals dying in hunger.

Our results show that the water content of normal adrenals varied slightly, but was never greater than 73% in old females, 74% in old males, and 71% in younger males. In starved rats enlarged adrenals almost invariably showed a greater water content, usually about 76 to 77%, the maximum found being 84%. Marrian reported similar findings in pigeons (18).

The ratio between the solid content of such adrenals and the original body weight did not appreciably change from normal, indicating that there had been no real loss of solid, nor any gain. This is in agreement with the findings of Addis, Poo, and Lew (1) that rat adrenals after a seven day fast showed no loss or gain in protein content. Data in the literature concerning adrenal lipides in starvation give no definite clue to any over-all lipide change (24, 28, 31), though there is possibly some depletion (25).

Nuclear counts of specified areas in sections of adrenals enlarged from starvation showed fewer nuclei than normal, apparently an indication of fewer and larger cells per unit area. The nuclei also tended to be larger. Such sections showed a narrower glomerular zone, and some evidence of degeneration of the reticular zone (cf. 15).

The change in water content, with unaffected solid content, indicates that adrenal enlargement resulting from acute starvation is due to an increased

uptake of fluid by the cells. *It is not a hypertrophy, but is perhaps in the nature of a hydropic degeneration.*

Perhaps bearing on this point are the observations of Mulinos and Pomerantz (22) that peritoneal tissues adjacent to the adrenals often become oedematous in such animals, and those of Barlow and Whitehead (2) that in such acute starvation there is evidence of a general hydraemic plethora.

(An opposite condition, increase of the solid content of the adrenals of rabbits chronically poisoned by ammonia, has been reported by Fazekas (8).)

Edelmann (7) has demonstrated that a significant enlargement of the adrenals of rats subjected to anoxia through decreased pressure is due to a hyperaemia.

The normal rat adrenal, as seen in our experiments, is yellow, dull yellow, or greyish yellow, sometimes with a faintly reddish tinge. In one or two cases (cf. Table III) in apparently normal animals the adrenals were dark grey or greyish and unduly large; they possibly were not normal. Glands enlarged through starvation were almost invariably dark grey, or what is best described as dark dirty grey.

This change in colour, accompanied by increase in water content of the cells, indicates that in the final stage of starvation (but only then) a pathological process sets in in these glands. In extreme cases it probably develops to a marked degeneration, as indicated by the first two asterisked cases in Table III, in which, when the glands were punctured, free fluid exuded. Unfortunately such extreme cases cannot be produced at will experimentally.

Whether the onset of this pathological condition can be related to the earlier increased activity cannot be stated.

It seems probable that whenever experimental treatment results in marked discoloration of the adrenals, this indicates presence of a pathological process. Mulinos and Pomerantz (22) stated that those of their starved rats "were large and red with a cortex that was friable and congested with blood." It is to be remembered that water was withheld from their animals.

Colour changes resulting from other treatment have been recorded. Thus McCarrison (17) noted that pigeons on a sole diet of milled and autoclaved rice developed enlarged yellow-red or more commonly red-brown adrenals (cf. also Findlay (9) ). The condition of B-complex deficiency in pigeons shows, indeed, a very close parallel to that in starved rats. Marrian (18) demonstrated an increased water content of the adrenals, and McCarrison (17) stated that equal areas of adrenal sections showed fewer cell nuclei than normal. Hence in this condition also something in the nature of a hydropic degeneration may be postulated.

Selye (26) found that in rats poisoned with formaldehyde or some other drug or subjected to severe exertion or to cold the adrenals became enlarged and dark brown. Glock (10) very recently reported that in the terminal stages of fatal poisoning by thiourea or thiouracil rat adrenals were frequently enlarged and dark reddish brown in colour. We have ourselves observed a similar change following chronic dosage with oestradiol (5).

## Acknowledgments

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# DETERMINATION OF THE EFFECT OF IRRADIATION BY X-RAY ON *p*-AMINOBENZOIC ACID, USING *CLOSTRIDIUM ACETOBUTYLICUM* FOR ASSAY[1]

By Ivan C. C. Tchaperoff[2]


## Abstract

A study has been made on the effect of X-ray irradiation by producing inactivation of *p*-aminobenzoic acid as determined by assay with *C. acetobutylicum*. It has been shown that *p*-aminobenzoic acid in concentration of $1 \times 10^{-4}$ μgm. per cc. is inactivated by an X-ray dose as small as 50 *r* at 190 kv. The significance of the destruction of Paba in connection with the treatment of infective lesions is discussed.


The importance of *p*-aminobenzoic acid (Paba) as a bacterial growth factor is to-day well established. According to the criteria enunciated by Fildes (2) Paba is a substance probably concerned in the metabolism of most bacteria and one that may possibly play a role in the cellular metabolism in general. The extraordinary biological activity of this compound as a bacterial growth factor suggests that it may be chemically related to a co-enzyme. Park and Wood (7) and Rubbo and others (8) have proved conclusively that Paba functions as a bacterial growth factor for *Clostridium acetobutylicum*, which is unable to produce this metabolite from the media in which it grows.

In this report it is demonstrated that the destruction of Paba by X-ray irradiation can be used as a means of producing bacteriostasis in a way similar to the mechanism by which sulphonamides are believed to act.

Tchaperoff (9) has reported that sulphonamides and Paba in concentrations of the order of 10 mgm. per 100 cc. are broken down by very heavy doses of irradiation. The breakdown was measured by Marshall's test. As described by Marshall (5) this test cannot be used for measuring solutions lower than 0.5 mgm. using the Klett Biological colorimeter. It was therefore decided to use the method described by Park and Wood (7) to investigate the breakdown following irradiation of Paba in concentrations of $1 \times 10^{-4}$ μgm. per cc.

## Materials and Methods

*Clostridium acetobutylicum* 862, obtained from the American Type Culture Collection, grows well anaerobically on an infusion of corn meal. The corn meal infusion tubes are autoclaved for one hour at 15 lb. pressure before inoculation. After inoculation the tubes are evacuated and sealed by a blow torch. Daily transfers are made to fresh corn meal tubes so that very vigorous growth is obtained in 24 hr.

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*Contribution from the Associate Committee on Army Medical Research, National Research Council of Canada.*

[2] *Major, R.C.A.M.C.*

The basal medium used is the same as that described in papers by Park and Wood (7) and Oxford *et al.* (6). It consists of: glucose, 20.6 gm.; asparagine, 1.0 gm.; sodium chloride, 0.01 gm.; ferrous sulphate heptahydrate, 0.01 gm.; manganese chloride tetrahydrate, 0.01 gm.; reduced iron, 3 to 5 mgm.; distilled water, 1000 cc. The glucose is made up separately into solution and added to the rest of the basal medium just before use. Both solutions are kept as stock solutions at four times the above concentration. They are sterilized by autoclaving for 15 min. at 15 lb. pressure.

The *C. acetobutylicum* is transferred from corn meal tubes to the basal medium enriched with 1.0% peptone. On this medium, growth takes place vigorously in 36 to 48 hr. in test-tubes that are evacuated and sealed as for the corn meal tubes. Any transfer of corn meal is avoided by three to four serial transfers on the peptone medium. All incubations are at 37° C. From a vigorous growth on the peptone enriched medium 30 cc. of the culture is centrifuged, the supernatant is discarded, and the residue suspended in 20 cc. of basal medium. As described by Park and Wood, this is repeated three times to get rid of any peptone that may be carried over. Final suspension is made up in 20 cc. of basal medium for inoculation.

Crystalline biotin supplied by Merck and Co. is made up to $1.5 \times 10^{-4}$ $\mu$gm. per cc. in distilled water. Park and Wood have shown that biotin is necessary for growth on this basal medium in concentration of at least $1.5 \times 10^{-5}$ $\mu$gm. per cc.

Chemically pure Paba supplied by Eastman Kodak is made up to $1 \times 10^{-3}$ $\mu$gm. per cc. in distilled water. Park and Wood have shown that Paba is extremely active as a growth factor in concentrations of $1 \times 10^{-2}$ to $1 \times 10^{-5}$ $\mu$gm. per cc. Great care has to be taken in cleaning all glassware as at first contradictory results, which were due to Paba contamination, were obtained.

Paba in a concentration of $1 \times 10^{-3}$ $\mu$gm. per cc. and biotin $1.5 \times 10^{-4}$ $\mu$gm. per cc. were irradiated separately in sealed glass test-tubes with the following factors: −190 kv., 20 ma., 4 mm. tin filter, 40 F.S.D.* Test-tubes in pairs containing Paba and biotin solutions were given 50, 100, 150, 200, 400, 800, and 1200 *r* respectively. As will be seen later the Paba is destroyed.

The method of using a McIntosh and Fildes anaerobic jar was found unsatisfactory for growth over 48 hr. Contaminations appear to take place from the 'moist oats' method described by Park and Wood. Modified Turnberg tubes proved a very satisfactory solution of the problem. They are made to fit exactly into the Evelyn photoelectric colorimeter, which is used for measuring the opacity of the growth. The tubes have an added constriction made one inch below the tube neck. This space was packed with a cotton wool plug to prevent contamination of the medium. The tubes are of sufficient

* *Focus Skin Distance.*

length so that 20 cc. of medium fills them only one-third. A second glass trap packed with cotton wool is attached to the side arm outlet of the tube. After the tubes are filled with the medium, sterilized, and inoculated, they are evacuated by the vacuum pump and the vacuum renewed every 24 hr. When it is desired to measure the degree of opacity of the growth, the tubes are placed directly in the colorimeter. This obviates the necessity of removing at least 5 cc. samples each day for determination of growth and lessens the chances of contamination.

Each tube contained: 5 cc. of the stock solutions of glucose in quadruple strength; 5 cc. of the stock solution of salts in quadruple strength; 5 cc. of the biotin solution to give a final concentration of $1.5 \times 10^{-5}$ μgm. per cc.; and 5 cc. of the Paba solution to give a final concentration of $1 \times 10^{-4}$ μgm. per cc., thus making up 20 cc. in each tube of the required concentrations. Table I shows the contents of the tubes and the amount of radiation in "*r*" given to the biotin and Paba solutions before final dilution. The tubes were inoculated with 0.5 cc. of the *C. acetobutylicum* inoculum, which had been suspended, as described, in 20 cc. of basal medium free from peptone, biotin, or Paba. It was found necessary to use the inoculum when it was growing very vigorously otherwise it died out in the tube. Park and Wood also found it necessary to use as large an inoculum as 0.5 cc.

The tubes were incubated at 37° C. and the degree of opacity measured each day by placing the tubes directly in the Evelyn photoelectric colorimeter.

TABLE I

GROWTH OF *C. acetobutylicum* ON BASAL MEDIUM

| Addition to basal medium | Radiation in *r* | Tube No. | Time in days | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Radiated Paba + radiated biotin | 50 | 1 | 93 | 93 | 92 | 92 | 92 | 92 | 92 | 82 | 82 |
| | 50 | 2 | 93 | 93 | 92 | 92 | 92 | 92 | 92 | 82 | 82 |
| | 100 | 3 | 95 | 95 | 92 | 92 | 92 | 92 | 91 | 73 | 71 |
| | 100 | 4 | 94 | 94 | 90 | 90 | 90 | 90 | 90 | 70 | 70 |
| | 150 | 5 | 91 | 91 | 89 | 89 | 89 | 89 | 89 | 70 | 79 |
| | 150 | 6 | 93 | 93 | 90 | 90 | 89 | 84 | 81 | 71 | 70 |
| | 200 | 7 | 93 | 93 | 90 | 91 | 91 | 91 | 91 | 82 | 80 |
| | 200 | 8 | 92 | 92 | 91 | 91 | 91 | 91 | 91 | 82 | 80 |
| | 400 | 9 | 90 | 90 | 90 | 89 | 89 | 87 | 89 | 75 | 78 |
| | 400 | 10 | 91 | 91 | 90 | 90 | 90 | 91 | 91 | 78 | 80 |
| | 800 | 11 | 90 | 90 | 90 | 90 | 88 | 80 | 78 | 67 | 65 |
| | 800 | 12 | 91 | 91 | 89 | 89 | 89 | 89 | 89 | 82 | 82 |
| | 1200 | 13 | 93 | 93 | 90 | 89 | 89 | 89 | 89 | 82 | 80 |
| Normal Paba + normal biotin | Nil | 14 | 89 | 89 | 89 | 37 | 37 | 42 | 35 | 35 | 36 |
| | Nil | 15 | 91 | 91 | 83 | 43 | 42 | 42 | 45 | 42 | 45 |
| No Paba and no biotin | Nil | 16 | 91 | 91 | 89 | 89 | 89 | 82 | 77 | 70 | 76 |
| | Nil | 17 | 90 | 90 | 89 | 89 | 89 | 88 | 72 | 74 | 72 |

NOTE: *Opacity measured by Evelyn photoelectric colorimeter (base line 60).*

## Results

The degree of opacity produced by bacterial growth in each tube is shown in Table I; as the growth increases the readings decrease. The effect of radiated Paba and radiated biotin had been added and demonstrates the destruction or inactivation of the Paba by irradiation. Table II shows no

TABLE II

GROWTH OF *C. acetobutylicum* ON BASAL MEDIUM

| | Time in hours | |
|---|---|---|
| | 48 | 64 |
| Basal medium only | 0 | 0 |
| Basal medium + Paba + biotin | 0 | +++ |
| Basal medium + radiated Paba + radiated biotin | 0 | 0 |
| Basal medium + radiated Paba + biotin | 0 | 0 |
| Basal medium + Paba + radiated biotin | 0 | +++ |

NOTE: *No growth indicated by 0; marked growth indicated by +++.*

evidence of destruction of the biotin and the whole change is due to the inactivation of the Paba. As all the tubes containing radiated Paba show such a close comparison the results are averaged in Table III from which Fig. 1 is constructed. The growth on basal medium with Paba and biotin

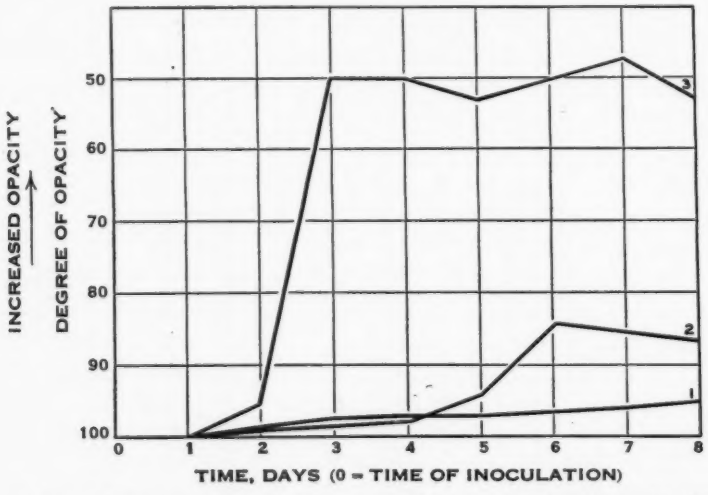


FIG. 1. *Curve 1 shows growth of C. acetobutylicum on basal medium with addition of radiated Paba and radiated biotin. Curve 2 shows growth of C. acetobutylicum on basal medium without Paba or biotin. Curve 3 shows growth of C. acetobutylicum on basal medium with addition of Paba and biotin.*

TABLE III

GROWTH OF *C. acetobutylicum* ON BASAL MEDIUM

| | Time in days | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Average figure of readings from tubes containing *radiated biotin* and *radiated Paba* at 195 kv., 50–1200 *r*. | 92.2 | 90.4 | 90.4 | 90.3 | 90 | 89 | 88.8 |
| Average figure of readings from tubes containing *unradiated biotin* and *Paba* | 90 | 90 | 86 | 40 | 39.9 | 42 | 40 |
| Average figure readings from tubes containing only basal medium without *biotin* or *Paba* | 90.5 | 90.5 | 89 | 89 | 89 | 85 | 74.5 |

NOTE: *Opacity of growth measured by Evelyn photoelectric colorimeter (base line 60).*

(Curve 3, Fig. 1) reaches nearly the maximum at the third day, whereas little or no growth occurs on the basal medium with radiated Paba and radiated biotin (Curve 1, Fig. 1). As control, Curve 2 (Fig. 1) shows the growth on basal media without the addition of Paba or biotin.

## Discussion

The results demonstrate that as little as 50 *r* can inactivate Paba as a growth factor when radiated in a concentration of $1 \times 10^{-3}$ μgm. per cc. and diluted to $1 \times 10^{-4}$ μgm. per cc. in the basal medium. This is well within the known concentration for the demonstration of the biological activity of Paba by *C. acetobutylicum*. It is important to radiate the Paba as nearly as possible at the concentration in which it will occur in the medium, if changes are to be produced by small doses of irradiation. The fact is brought out by Dale (1) in discussing the effects of X-rays on enzymes. Dale irradiated carboxypeptidase and showed that X-rays have a qualitatively uniform effect on enzymes over the whole range from very small doses to doses 1000 times as large—(400 to 400,000 *r*). Fricke and Hart believe that the changes are produced by "X-ray activated water" and that it is in the nature of an oxidation process. Hydrogen peroxide is hypothetically believed to be formed but exists in the solution only momentarily.

The method used by Park and Wood, which has been followed in this report, is only semi-quantitative for *p*-amino acid. It is desirable to repeat the experiments using the more quantitative method of Lampen and Peterson (3) with an assay range 0.3 to 1.5 mμgm. of Paba, the error being 10% or that of Lewis (4) with a sensitivity range of 0.15 to 0.5 mμgm. of Paba. The latter uses an aerobic culture method and the acid produced in the medium is titrated with 0.1*N* sodium hydroxide using bromothymol blue as an indicator.

Park and Wood give the minimum concentration of Paba in the basal medium at which *C. acetobutylicum* will grow as $1 \times 10^{-5}$ μgm. per cc.; our

experiments indicate that the irradiation reduces the Paba from $1 \times 10^{-4}$ to below $1 \times 10^{-5}$ μgm. per cc.

It is believed that the demonstration of the destruction by Paba by irradiation indicates the part played by X-rays in the treatment of infection. X-ray treatment makes it possible to treat infected areas intensely since the irradiation in the doses used in treatment has no systemic effect. Because it has been demonstrated that Paba can be destroyed by irradiation, it is suggested that X-ray treatment is preferable to sulphonamides in infections that are sulphonamide resistant and that produce large amounts of Paba either by synthesis or by freeing it from the tissues.

It remains to be demonstrated whether irradiation can reduce the Paba concentration in suitable basic media faster than the organism can produce it and thus can produce bacteriostasis. Such a demonstration would offer a reason for the use of X-ray therapy in the treatment of infective lesions.

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The radiation of the chemical substances was made in the X-ray Department of the Toronto General Hospital by permission of Dr. G. E. Richards.

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# COLLECTING AND HANDLING MOSQUITOES ON WESTERN EQUINE ENCEPHALITIS INVESTIGATIONS IN MANITOBA[1]

By J. McLintock[2]


## Abstract

Mosquitoes used for virus analysis in Manitoba are obtained by means of hand catches or by the use of light traps, sometimes supplemented with dry ice. A trap is described that has been used successfully for this purpose during the past three years. This trap is simple to operate, the insects are taken in good condition for identification, and newly emerged specimens are killed, thereby eliminating the majority of those that could not possibly be infected with the virus of western equine encephalitis. The methods of shipping, sorting, identifying, and storing specimens are given. These methods are, in large part, standard entomological procedures adapted to present needs.


During the past four years the Manitoba Department of Health and Public Welfare, in conjunction with the Children's Hospital of Winnipeg, has carried on an investigation into the possible role of vectors in the spread of western equine encephalitis in the province. Thus far, attention has been concentrated on mosquitoes as the most likely suspects. In order to discover whether these insects are carrying the virus in nature it has been necessary to obtain large numbers of living specimens for virus analysis. For this purpose a trap was required that would attract a fairly large sample of the mosquito population, that would keep the mosquitoes alive until they reached the laboratory (for 24 to 48 hr.), and in good condition for identification. In addition, since the traps would be serviced by laymen, their operation had to be as simple and convenient as possible.

Two traps for a similar purpose have been designed by Reeves and Hammon (7) but these were intended to be operated under somewhat different conditions from those existing in Manitoba. One of their traps utilizes a light and carbon dioxide vapour as attractants, but the light was restricted to a 40° angle and in the morning the entrapped mosquitoes had to be removed by hand while the trap was still in the field. The other was a modified New Jersey trap in which the killing jar was replaced by a 14 in. square cage with the addition of a dry ice stimulus. Regarding this latter trap, the authors point out that, beside a fairly high mortality (36.2% compared with 20.7% in the "live trap"), "a large proportion of the 'living mosquitoes' in the standard trap were unsatisfactory for feeding experiments or other biological work, and their period of survival was often times brief". I believe that the trap described below has overcome most of the preceding difficulties, at least for the temperate climate of Canada. Dry ice can also be used with this trap and as is well known (3, p. 10; 7) greatly increases the proportion of females in the catch.

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*Contribution from the Virus Laboratory of The Department of Health and Public Welfare for the Province of Manitoba and of the Children's Hospital of Winnipeg, Winnipeg, Man.*

[2] *Entomologist.*

In the early stages of this investigation a modified version of Essig's (1) gnat trap was tried. In this trap an electric light attracted the insects and an electric fan drew them down into a cloth bag held distended by a wire frame. Although it attracted, trapped, and held the mosquitoes, and was simple to operate, this trap had the serious disadvantages that a large proportion of the specimens were bruised beyond recognition and the mortality was too high, sometimes reaching 60%. This was due to the specimens having to pass through the blades of a rotating fan on their way into a bag in which the resting surface was too small for the larger catches and which could not be protected from a certain amount of buffeting by the wind nor from heavy rains. After further experimentation to circumvent these difficulties, and following some suggestions made by Dr. W. McD. Hammon, the trap described below was designed and found to be quite satisfactory.

Since viruses will only propagate in living tissues, the problems of handling those tissues in such a way as to retain the virus with a sufficient titre for experimentation and of keeping the tissues susceptible to the virus, become of major importance in any investigation involving the aetiology of virus diseases. This fact has led the numerous authors of the growing literature on equine encephalitis to describe in some detail their methods of handling infected and possibly infected material. In those papers dealing with the role of mosquitoes in the transmission of the virus encephalitides it so happens that the investigations have either been carried out in climates considerably warmer than that of central Canada (the United States, Japan, Africa) or the reports have been published in the relatively inaccessible Russian literature. In central Canada it has been our experience that many of the methods used in warmer climates are wholly unsuited to conditions prevailing in the Prairie Provinces. As far as I am aware there is no previous report of living mosquitoes, taken in light traps, having been shipped to the laboratory for identification and preservation. Since this procedure has had to be followed in Manitoba during the past four years, it seems advisable to communicate the methods that have been successful in detecting infected mosquitoes in nature (6). No claim to originality is made for the entomological procedures involved, most of them having been borrowed from practices followed in other lines of entomological investigation and adapted to present needs.

## The Trap and Its Operation

In so far as this trap utilizes an electric light to attract the insects and the suction of a fan to draw them in, it can be considered as a modified New Jersey trap. In the course of this investigation six of these traps have been located at different points in the province and, apart from minor mechanical breakdowns, have been in continuous operation during three seasons.

The trap assembly (Fig. 1) consists of three separate units, (1) a cylinder containing an electric motor and fan, (2) a funnel and canopy carrying a light, and (3) a cage that fits into the cylinder and holds the entrapped insects. The cylinder, of No. 24 gauge sheet iron, is about the size of a small garbage

can 23½ in. high and 12 in. in diameter. This cylinder is supported at a height of 10½ in. above the ground by three wooden legs bolted to its wall. The bottom 9¾ in. of the cylinder is occupied by a 1/15 or 1/4 h.p. electric motor carrying

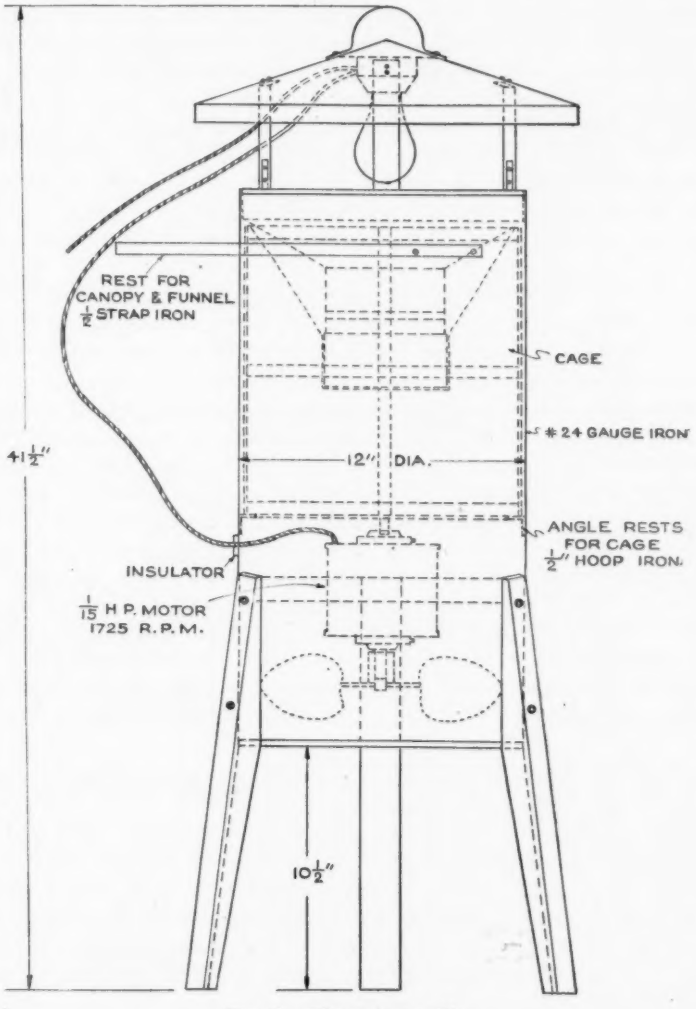


FIG. 1. *The trap assembly.*

a 10 in. exhaust fan, which it rotates at approximately 1725 r.p.m. The funnel (Fig. 2), of the same gauge iron, is made to fit into the top of the cylinder. This funnel tapers to a collar 5 in. in diameter and 2¾ in. long. A metal, cone-shaped canopy 16 in. in diameter covers the whole trap and is

supported by three legs of $1\frac{1}{4}$ in. hoop-iron spot-welded to the wide rim of the funnel and to the roof of the canopy. This places the bottom rim of the canopy $2\frac{3}{4}$ in. above the funnel and the top of the cylinder. A standard lamp socket to carry the light is attached to the inside of the apex of the canopy.

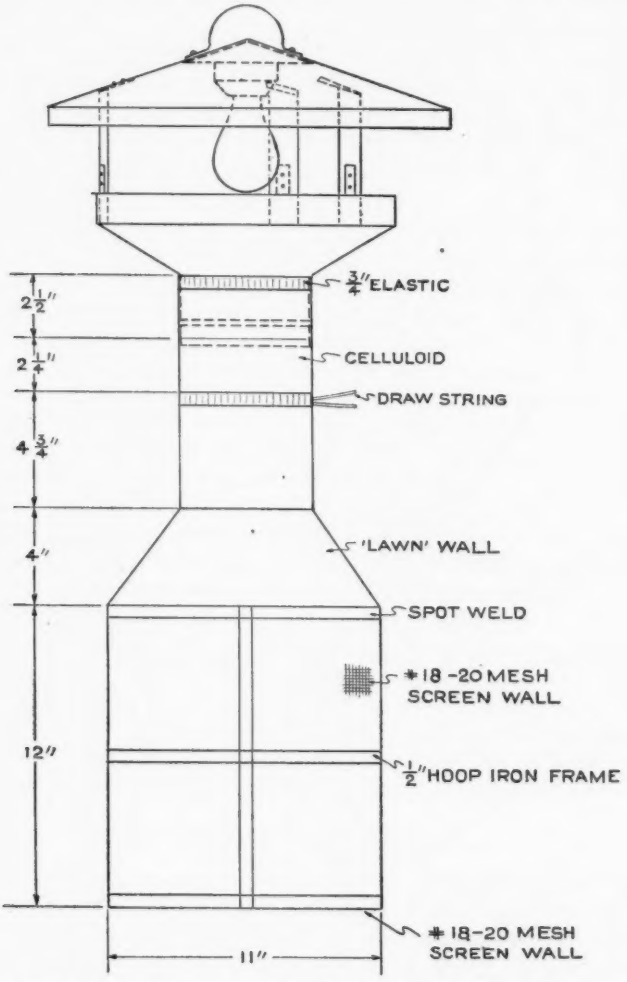


FIG. 2. *Sleeve of cage extended and attached to funnel and canopy.*

The canopy, lamp socket, and funnel form one unit, and the wires connecting lamp and motor in parallel are left long enough to allow this unit to be removed from the top of the cylinder by means of a handle attached to the top of the canopy. The top of the funnel when in position is held flush with the top

of the cylinder by means of three ½ in. hoop-iron cleats attached to the legs that support the canopy.

The body of the cage (Fig. 2) is a cylinder of 18-mesh galvanized or "galvanoid" wire screening, 11 in. in diameter and 12 in. high. The screening is supported on a ½ in. hoop-iron frame consisting of three horizontal circles and four vertical strips and also covers one end of the cylinder. The seams where the screening joins are soldered. The top of the cage is made of the cotton material known as 'lawn', which is sewn to the top rim of the screen cylinder from where it tapers for four inches to a diameter of 5¼ in. and from there on is continued as a sleeve 9½ in. long. At the end of this sleeve a ¾ in. elastic collar is sewn into the cloth and 2½ in. below this a celluloid collar 2¼ in. wide is also sewn into the cloth. A draw-string is inserted just below the celluloid. This construction allows the lawn sleeve to be 'telescoped' into the body of the cage. In the telescoped position the celluloid collar is near the bottom of the cage and is rigid enough to keep the aperture open. The draw-string permits the cage to be closed before removal from the trap and the elastic band attaches the lawn sleeve to the 5 in. collar of the trap funnel.

To set the trap, the funnel and canopy are removed and the cage placed inside the trap cylinder; three metal cleats support the bottom of the cage just above the fan motor, which places the top of the cage about 1½ in. below the top of the cylinder. The cage is then attached to the trap funnel by means of the elastic band at the end of the cage sleeve and the funnel placed in the top of the cylinder. In so doing the cage sleeve is telescoped into the cage. The current is then turned on. The insects are attracted by the 50 watt light under the canopy and are drawn down into the cage by the suction created by the fan. The telescoping arrangement of the sleeve provides an eddy-space at the top of the cage where the smaller insects such as mosquitoes can rest without being damaged by the larger insects that are drawn into the trap with them. It should be noted however that the suction of the fan is not strong enough to draw in insects any larger than cutworm moths or June beetles.

In the morning, a wad of rags is stuffed into the mouth of the funnel to prevent any of the entrapped insects from escaping when the fan motor is stopped. The funnel and canopy are then raised and rested on the edge of the cylinder while the draw-string is being pulled tight to close off the cage. The cage can then be lifted from the trap.

To obtain best results with the trap it is important that it be stopped and emptied as soon as possible after daybreak as the heat of the sun raises the temperature inside the trap with a consequent fatal lowering of the relative humidity. For this reason too the traps should be placed in locations that are shaded as much as possible from the rays of the rising sun. A 10 in. exhaust fan at 1725 r.p.m. moves about 150 to 200 cu. ft. of air per minute through a 5½ in. duct. This was found to have a considerable desiccating effect on the male and newly emerged mosquitoes. Mortalities as low as 17% (out of 1770 specimens) and 18% (out of 2460 specimens) were obtained for female mos-

quitoes under the best conditions of operation using only the light as attractant. During three successive nights when the weather was cloudy and cool and the light was supplemented with dry ice, 2862 specimens (all females) were obtained with a mortality of only 0.7%. However the mortality never went below 49% (out of 851 specimens) for the males. The mortality among newly emerged mosquitoes was determined by running the trap for several nights beside a slough during an emergence; here the mortality was 81% of both sexes combined (out of 327 specimens) and this was during a cool wet week. This selectivity is a decided advantage in collections made for the purpose of obtaining specimens for virus analysis. Only those specimens that have had a blood meal will be carrying virus and by the time a mosquito is ready to feed, its integument is already hardened and relatively impermeable. Since only living specimens are preserved or used for virus analysis, the trap eliminates a large number that would not be carrying virus.

In using dry ice, about 5 or 6 lb. are placed in a cloth bag and tied to the top of the canopy of the trap. The gas falls over the edge of the canopy and is drawn through the trap to be blown out at the bottom by the fan. An almost pure catch of female mosquitoes is obtained when dry ice is used.

Although this trap was designed primarily for trapping mosquitoes, it could obviously be used with equal success for trapping living specimens of other small insects that are attracted to light.

## Hand Collecting

Hand collections are made by means of the aspirator described by Moore (5) or with the collecting bottle described by Mail (4). Suction for using the aspirator in the field is supplied by the intake manifold of a car and about 100 ft. of rubber tubing is sufficient to reach the inside walls of barns, sheds, stables, root houses, and other resting places. The amount of suction is controlled by means of a screw clip attached to the tubing about one foot from the aspirator. The collecting bottle is used only for picking the mosquitoes from horses and cattle in locations that cannot be reached by car. For collecting mosquitoes the aspirator has the advantage of holding a larger number of insects than the usual tube operated by mouth suction, also there is no possibility of the mosquitoes escaping once drawn in. When the storage tube of the aspirator, or the bottle, is full, it is emptied into the trap cage described above; each cage contains the collections from only one locality.

## Shipping, Sorting, Identification, and Preservation of Specimens

The traps are serviced mainly by volunteer workers who send in the cages containing each night's catch, with a card showing the collection data, to the laboratory in Winnipeg. The cages are shipped via railway express in cardboard cartons 12 × 12 × 14 in. The bottom of each carton is lined with heavy waxed paper on top of which sits a 1 in. cellucotton pad kept moist with water. The cage rests on top of the pad. By this means the catches reach the laboratory never more than 48 hr. after being removed from the

traps. Counts made on hand catches shipped by this method have indicated that not more than 5% of the mortality is due to this procedure.

On arrival in the laboratory each cage is removed from its container, given a code number, and the collection data entered in a log book opposite the code number.

When a cage is held under a light the mosquitoes inside are easily seen through the wire screening and are removed from the cage by means of the aspirator mentioned above. In this case the aspirator is merely inserted through the sleeve of the cage. In the laboratory, suction for the aspirator is supplied either by a filter pump or by a standard laboratory vacuum pump. While the aspirator is held in one hand the rubber tubing is pinched in the other and only opened to pick up a mosquito. When the storage tube is full the aspirator is removed from the cage and disconnected from the rubber tubing. A finger is then placed over the end of the glass tubing to which the rubber tubing was attached and two or three drops of ether poured down the intake tube of the aspirator, care being taken that no liquid ether enters the storage tube. In a few minutes, when the insects have ceased moving, they are emptied into a 4 oz. pomade jar and covered with a piece of marquisette. A small wad of cotton and a vial of ether are kept handy on the bench where the identifications are being made and if the mosquitoes show signs of becoming active before they are identified, the cotton soaked with ether is held over the jar for a few minutes.

While still under the ether the mosquitoes are removed from the jar with forceps and identified under a microscope. As each specimen is identified it is placed in a 10 cc. ampoule, which is used for storage. As a rule only one species from one locality is placed in each ampoule and no less than 30 specimens to an ampoule. When the catches are small or contain a few specimens of the rarer species, either the same species from different localities are pooled or each of these species is isolated in jars covered with marquisette and containing a moistened pad of cellucotton; these are held until sufficient specimens are obtained to make up a quota for an ampoule. The stem of a 10 cc. ampoule is large enough to permit easy entry of a relaxed, anaesthetized mosquito, but too small to allow a flying mosquito to escape. When each ampoule has received its quota, it is sealed by quickly drawing out the stem in the fine flame of a blast burner, then given a code number and placed in a flat cardboard box lined with cotton. These boxes are stored on top of a block of dry ice kept in an ice chest.

For some time all living female mosquitoes taken were preserved intact for virus analysis. However Hammon, Reeves, and Izumi (2) have suggested the possibility that the comparatively fresh blood in the abdomens of newly engorged specimens might contain antibodies in sufficient titre to neutralize the virus in an inoculum prepared from wild-caught mosquitoes. Recently we have adopted their practice of crushing the abdomens of such specimens for smears for the precipitin test, the remainder of such engorged specimens being added to the non-engorged.

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# RECOVERY OF A STRAIN OF WESTERN EQUINE ENCEPHALITIS VIRUS FROM *CULEX RESTUANS* (THEO.) (DIPTERA: CULICIDAE)[1]

By Marjorie Norris[2]

## Abstract

Since an epidemic of western equine encephalitis in Manitoba in 1941, attempts have been made each year to isolate the virus from mosquitoes collected during the summer months. In 1942, 94 pools of mosquitoes were tested: 43 of the genus *Aedes*, 36 of *Culex*, and 15 of *Culiseta*. Suspensions made from each pool, which consisted of from 1 to 30 insects, were injected intracerebrally into guinea pigs. No isolation of virus was made. In 1943, 69 pools were tested: 49 of *Aedes*, 6 of *Culex*, 1 of *Culiseta*, 1 of *Anopheles*, and 12 of mixtures of different genera. Each suspension was derived from 10 to 75 mosquitoes and was injected intracerebrally into Rockefeller strain Swiss mice. There was no recovery of virus. In 1944, 90 pools were tested: 10 of *Culex*, 74 of *Aedes*, and 6 of *Culiseta*. Suspensions were usually made from 70 to 100 insects, and injected intracerebrally into Swiss mice of the Rockefeller strain. From one pool of *Culex restuans* (Theo.), a strain of western equine encephalitis virus was recovered.

Since Kelser (16) in 1933 showed that the mosquito *Aedes aegypti* could transmit the virus of western equine encephalitis under laboratory conditions, numerous other investigators have reported laboratory transmissions of this virus by other species of the genus *Aedes*. Their work has been summarized by Hammon and associates (12). The first report of the recovery of the virus from a naturally infected *Aedes* mosquito has been made recently by Hammon and Reeves (10).

Previous to 1941 *Culex* mosquitoes had not been implicated as vectors of equine encephalitis viruses. In that year Hammon and associates (14) reported the isolation of one strain of *W.E.E.** virus from *C. tarsalis* Coq. collected in the Yakima Valley, Wash., where annual epidemics of the disease occur. Shortly afterwards the recovery of four additional strains from the same species of mosquito was reported by the same group of workers (13). Later Hammon and Reeves proved that this species could act as a vector under experimental conditions (11). Transmission was also reported with *Culiseta inornata*, a mosquito that these workers had also found infected in nature.

The presence of *W.E.E.* virus in Western Canada was established. Fulton (3) had first isolated the virus from the brain of a horse during the epidemic of 1935 in Saskatchewan. In 1938, the same investigator (4) recovered this virus from a human brain during an epidemic amongst humans and horses in that province. In Alberta, Gwatkin and Moore (6) had shown the possible

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* *W.E.E. = western equine encephalitis.*

presence of *W.E.E.* virus in the brains of ground squirrels. Later Gwatkin (5) and Gwatkin and Moynihan (7) reported an unsuccessful search for carriers of equine encephalitis virus in a number of small mammals, birds, and a few insects.

Interest in the role of the mosquito as a possible vector of *W.E.E.* in Manitoba began in 1941 when the first epidemic of this disease in man in this province occurred, 509 cases being reported. Adamson and Dubo (1) reported their findings in 212 clinical cases. Serological tests were done on 125 of these, 55 being found to contain antibodies against *W.E.E.* virus. The etiological agent was further proved by the recovery of the virus from the spinal fluid of one patient (2). The same summer, during an epidemic in Alberta, Gwatkin and Moynihan (8) recovered a similar virus from the spinal fluid of a patient.

Following the epidemic in Manitoba, Mitchell and Pullin (17) made a survey of persons who had shown no clinical evidence of encephalitis. Their report showed that approximately 19% of blood specimens from 1013 persons contained neutralizing antibodies to *W.E.E.* virus.

In 1942 it was decided to attempt to isolate this virus from naturally infected mosquitoes. This report deals with the work from 1942 to 1944.

*1942*

Twenty-seven cases of encephalitis and 14 suspect cases were reported in the province. Complement fixation tests were done in this laboratory on blood specimens from all patients. Two were positive. Eighteen of the negative specimens were sent to Dr. J. Casals of the Rockefeller Institute, New York, who reported negative complement fixation tests for *W.E.E.* virus in all cases. Two negative specimens were sent to Dr. Herald R. Cox of Rocky Mountain Laboratory and six others to Dr. Wm. McD. Hammon of the University of California Medical Centre, for neutralization tests. With the exception of one specimen reported doubtful by Dr. Hammon, none was found to contain neutralizing antibodies for *W.E.E.* virus.

Mosquitoes were collected as described elsewhere*. They arrived at the laboratory alive and were identified and separated according to species before being made into suspensions for animal inoculation. Suspensions were made from 1 to 30 mosquitoes in 2.5 cc. of sterile normal saline. Each suspension was injected intracerebrally into two guinea pigs.

At first, in order to free the mosquitoes from bacteria, they were treated with ether for half an hour. It was soon found that suspensions made from mosquitoes artificially infected and then subjected to ether treatment, failed to infect mice when injected intracerebrally in dilutions of $10^{-1}$ to $10^{-4}$, whereas suspensions of other experimentally infected mosquitoes, not treated with ether, were fatal to all mice injected in similar dilutions. To remove the bacteria from the latter suspensions, they were centrifuged in an angle centrifuge at approximately 4000 r.p.m. for 20 min. Cultures on blood plates

* *McLintock, J. Can. J. Research, E, 24 : 55-62. 1946.*

showed them to be almost free of bacteria. Likewise when a mouse brain infected with *W.E.E.* was divided in two, one half, treated with ether, failed to infect mice, whereas the other half, untreated, killed all mice injected.

Following this discovery, bacterial contamination of all mosquito suspensions was reduced simply by centrifuging at 4000 to 5000 r.p.m. for 20 min. Very little difficulty due to contamination was encountered, owing, beyond doubt, to the small number of mosquitoes used for each suspension.

A total of 94 pools of mosquitoes was made for animal inoculation. These were composed of 38 pools of *Aedes vexans* (Mg.), 2 of *A. dorsalis* (Mg.), 3 of *A. flavescens* (Mul.), 34 of *Culex tarsalis* (Coq.), 2 of *C. restuans* (Theo.), and 15 of *Culiseta inornata* (Will.). Of these, 11 pools of *A. vexans*, 6 of *C. tarsalis*, and 1 of *C. restuans* had been treated with ether.

No recovery of virus was made. Since comparatively few cases of encephalitis were reported and only two of these gave positive serological tests, it is possible that few mosquitoes were infected that year. Of the 94 pools tested, 51 were of the genera most frequently found to be natural vectors, namely 36 of *Culex* and 15 of *Culiseta*. However, as few insects were used in the preparation of each suspension, the possibility of recovering a virus was greatly diminished: Hammon and associates (13) working with *Culex tarsalis* recovered one strain of virus from 386 mosquitoes taken during an epidemic. Since this article was written, Hammon, Reeves, and Galindo (Am. J. Hygiene, 42 : 299-306. 1945) have found an infection rate of 1 in 78 *C. tarsalis* taken during an epidemic period in Kern County, Calif., in 1943.

*1943*

In 1943, 10 cases of encephalitis were reported. Blood specimens were obtained from only four patients. Complement fixation tests for western equine virus done in this laboratory were all negative. Neutralization tests on these specimens gave one positive result, one doubtful, and two negative.

The mosquitoes were shipped alive to the laboratory. After identification the different species were placed in separate ampoules.

After visiting Dr. Hammon's laboratory*, it was decided to adopt the method described by him and his associates (15) for preparing mosquitoes for animal inoculation. The mosquito suspension is freed from contaminating organisms by being centrifuged in the angle head of an International multispeed attachment at 16,000 to 18,000 r.p.m. for 10 min. Unfortunately this multispeed attachment was not available during the summer collecting period, consequently it was necessary to store all mosquitoes to be used for virus analysis in the dry-ice chest until the work could be begun.

The work of animal inoculation was carried out in January and February, 1944, five to seven months after the mosquitoes had been stored. To prepare the suspension for inoculation, the ampoules of mosquitoes were removed from the dry-ice chest just before use. Suspensions of six pools, the capacity of the centrifuge, were usually made at one time. The ampoules were opened

* *I should like to express my sincere appreciation to Dr. Hammon for the privilege of studying, under his guidance, the methods used in his laboratory.*

and the contents turned out on gauze and washed with 25 to 30 cc. of sterile distilled water. They were then thoroughly ground in a mortar with alundum, and small amounts of 33% rabbit-serum broth were added till a total volume of 3.0 cc. had been used. The suspensions were centrifuged in the angle centrifuge at approximately 18,000 r.p.m. for 15 min. To hold the suspensions at as low a temperature as possible during this process, the angle head was kept in the refrigerator until used and approximately 3 lb. of dry-ice were placed in the metal casing of the centrifuge.

A portion of the clear liquid that lay under the usually fine surface layer of fat was removed by means of a Pasteur pipette, the end of the pipette being carefully inserted through an opening in the surface layer. Cultures of all suspensions were made on blood agar plates. In the majority of cases, they were found to be sterile; contamination, where it did occur, was very slight.

The experimental animal was changed to Rockefeller strain Swiss mice since Hammon and associates (15) had reported this strain to be more susceptible to western equine virus than guinea pigs. Under ether anaesthesia, five such mice*, three to four weeks old, were inoculated intracerebrally with 0.03 cc. of each suspension. The time required for the preparation of the suspensions and their injection into mice was 45 to 60 min. The mice were watched for 14 days for the development of symptoms of encephalitis before being destroyed.

Sixty-nine pools of mosquitoes were tested. These consisted of 47 pools of *Aedes vexans*, 1 of *A. campestris* (D.K.), 1 of *A. flavescens*, 6 of *Culex tarsalis*, 1 of *Anopheles maculipennis* (Mg.) (= *occidentalis* D.), 1 of *Culiseta inornata*, and 12 of mixtures of different genera. Each pool contained 10 to 75 insects.

No virus was isolated. Failure to recover virus may indicate its absence from the mosquitoes tested: the almost complete absence of encephalitis in man and horses suggests that there was little virus 'on the wing'. This year, as in the previous year, the number of insects used for each suspension was often small. In addition, 49 of the 69 pools tested consisted of *Aedes* mosquitoes, which have rarely been found to be vectors in nature.

*1944*

Twelve cases of encephalitis were reported. Blood samples were taken from five patients. Three gave positive neutralization tests.

This year, as usual, with the exception of the one week mentioned below, the mosquitoes used for animal inoculation arrived at the laboratory in a living state. Suspensions for injection were usually derived from 70 to 100 mosquitoes. When the number of a single species in one collection from a district was considered sufficient for animal inoculation, suspensions were made and injected into mice at once, as described above. When there were not enough mosquitoes for immediate injection, the living insects were placed in ampoules, according to species, and frozen. When enough of any one

* *The mice from which our colony was bred, were very kindly sent to us by the late Dr. L. T. Webster of the Rockefeller Institute, New York.*

species had been accumulated in this way from a particular district, they were pooled, and the resulting suspension injected into five Rockefeller strain Swiss mice.

In the latter part of July, a small epidemic of encephalomyelitis in horses was reported in the neighbourhood of Portage la Prairie, a town 60 miles from Winnipeg, where no collections were being made. A trap was set up at once at a spot one-half mile distant from a farm where cases of the disease had occurred, and daily collections of living specimens were shipped to Winnipeg. To increase the number of mosquitoes that could be tested for virus, a temporary laboratory was set up in Portage la Prairie where further trap and hand collections were identified, stored in ampoules, and frozen. Daily shipments of these specimens, well packed in dry-ice, were sent to Winnipeg. They were picked up immediately on arrival and stored in ice-chest until their injection into animals, frequently the same day. Injection of all pools into experimental animals was completed by the middle of October.

Ninety pools were tested: 61 of *Aedes vexans*, 8 of *A. flavescens*, 1 of *A. dorsalis*, 1 of *A. campestris*, 1 of *A. spencerii* (Theo.), 1 of *A. excrucians* (Wlk.), 1 of *A. cinerius* (Mg.), 4 of *Culex tarsalis*, 6 of *C. restuans*, 6 of *Culiseta inornata*.

Suspensions of living insects were made from 20 pools: 14 of *Aedes vexans*, 3 of *A. flavescens*, 2 of *Culex restuans*, and 1 of *Culiseta inornata*. The remaining 70 pools consisted of frozen specimens.

Forty-one pools were derived from the epidemic area around Portage la Prairie: 23 of *Aedes vexans*, 7 of *A. flavescens*, 2 of *Culex tarsalis*, 5 of *C. restuans*, and 4 of *Culiseta inornata*. Of the above, 12 pools of *A. vexans*, 3 of *A. flavescens*, 2 of *C. restuans*, and 1 of *C. inornata* consisted of living specimens. The number of pools and the number of specimens tested are shown in Table I.

From one pool of *Culex restuans* consisting of 100 insects, alive until the preparation of the suspension, the virus of *W.E.E.* was isolated. These mosquitoes were from a trap collection received from Portage la Prairie. They were not engorged so that the possibility of the virus being derived from a recent feeding on an infected animal may be discarded. From the epidemic area, a total of 426 specimens of this species was tested.

Identification of the virus was begun when, after three serial mouse brain passages, intracerebral inoculation was made into two guinea pigs, an animal known to be susceptible to western equine virus. Both animals exhibited a weakness of the hind legs on the second day after inoculation. The temperature of No. 1 was not obtained, that of No. 2 was 104.4° F. On the third day the temperatures were 104.4° F. and 105.0° F. and definite paralysis of the hind quarters had developed in both animals. The brain of No. 2 was removed and stored in buffered glycerine.

Passage of a suspension of the brain of guinea pig No. 1 was made to two other guinea pigs, Nos. 3 and 4. On the third day after inoculation the temperature of No. 3 was 105.0° F. and No. 4, 104.1° F. There was no sign of muscular weakness. On the fourth day the temperature of No. 3 had dropped

TABLE I

MOSQUITOES TESTED FOR THE PRESENCE OF ENCEPHALITIS VIRUSES

| Species | No. of pools from epidemic area | | No. of pools from other areas | | Total No. of pools | No. of specimens | | Total No. of specimens |
|---|---|---|---|---|---|---|---|---|
| | Frozen | Living | Frozen | Living | | Frozen | Living | |
| *Aedes vexans* | 11 | 12 | 36 | 2 | 61 | 4605 | 1457 | 6062 |
| *Aedes flavescens* | 4 | 3 | 1 | | 8 | 235 | 288 | 523 |
| *Aedes dorsalis* | | | 1 | | 1* | 47 | | 47 |
| *Aedes campestris* | | | 1 | | 1* | 64 | | 64 |
| *Aedes spencerii* | | | 1 | | 1* | 41 | | 41 |
| *Aedes excrucians* | | | 1 | | 1* | 15 | | 15 |
| *Aedes cinereus* | | | 1 | | 1* | 12 | | 12 |
| *Culex tarsalis* | 2 | | 2 | | 4 | 241 | | 241 |
| *Culex restuans* | 3 | 2 | 1 | | 6 | 290 | 191 | 481 |
| *Culiseta inornata* | 3 | 1 | 2 | | 6 | 438 | 95 | 533 |
| Total | 23 | 18 | 47 | 2 | 90 | 5988 | 2031 | 8019 |

* *These pools include specimens from the epidemic area.*

to 101.8° F. and paralysis of the hind legs was evident. The brain of this animal was removed and stored in buffered glycerine. On the same day the temperature of No. 4 had risen to 105.3° F., but there was still no sign of paralysis. This animal survived and its serum was later found to protect all mice against both dilutions of *W.E.E.* virus employed in the neutralization test.

The method used for the neutralization test was that described by Hammon and associates (9).

Titration of a 5% emulsion of our stock virus, the Rockefeller strain of *W.E.E.* virus, in 10% rabbit-serum broth, showed that a dilution of $10^{-6}$ was fatal to 95% of mice injected. This dilution and another $2 \times 10^{-5}$ ($\frac{1}{5}$ of the higher) were the two dilutions used in the test.

Titration of the newly isolated virus was done after eight serial mouse brain passages, when the constant incubation period and the fact that all injected mice died, indicated that the virus was becoming adapted to brain tissue.

Neutralization tests were then run with four human sera known to contain neutralization antibodies to *W.E.E.* virus, normal rabbit serum being used as a negative control. One of these sera was from a resident of the district

where the epidemic amongst horses had occurred; he had developed encephalitis during this outbreak and had recovered. Two other specimens were from patients from widely separated districts in the province. They had recovered from attacks of encephalitis during the summer. The fourth was from a laboratory worker vaccinated against the virus. All four specimens protected all mice against both dilutions of virus used. Titration of the virus had indicated that a dilution of $2 \times 10^{-5}$ of a 5% mouse brain suspension in 10% rabbit-serum broth caused the death of 95% of injected mice. This dilution and a second $4 \times 10^{-4}$ were the two employed in the test.

As a final step in the identification, three adult guinea pigs, whose serum was shown to contain no neutralizing antibodies for the Rockefeller strain of *W.E.E.* virus, were immunized by intraperitoneal injections of suspensions of the newly isolated virus. Injections of 1.0 cc. of a suspension of infected mouse brain in broth in dilutions of $10^{-5}$ to $10^{-2}$ were given at weekly intervals. A week after the last injection of this series 0.5 cc. of $10^{-1}$ dilution was given. This was followed a week later by 1.0 cc. of the same dilution. The last injection was repeated twice. Ten days after the final injection, the sera of all pigs showed the presence of neutralizing bodies for Rockefeller strain of *W.E.E.* virus.

TABLE II

INTRAPERITONEAL INJECTIONS OF MOUSE BRAIN INFECTED WITH *W.E.E.* VIRUS, GIVEN AT WEEKLY INTERVALS

| Dilution | Amount, cc. | Dilution | Amount, cc. | Dilution | Amount, cc. | Dilution | Amount, cc. |
|---|---|---|---|---|---|---|---|
| $10^{-5}$ | 1.0 | $10^{-3}$ | 1.0 | $10^{-1}$ | 0.5 | $10^{-1}$ | 1.0 |
| $10^{-4}$ | 1.0 | $10^{-2}$ | 1.0 | $10^{-1}$ | 1.0 | $10^{-1}$ | 1.0 |

I believe that this completes the proof that the virus recovered from *C. restuans* caught in nature in Manitoba in the summer of 1944 was the virus of western equine encephalitis, the same virus as that recovered in this laboratory from a patient in the summer of 1941. Work on mosquitoes as carriers of this virus in Manitoba is continuing.

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# MULTIPLE FEEDING HABITS OF SASKATCHEWAN MOSQUITOES[1]

By J. G. Rempel[2], W. A. Riddell[3], and Elspeth M. McNelly[4]

## Abstract

Precipitin tests are used to determine the source of the blood meal of Saskatchewan mosquitoes. The tests disclose that the *Aedes* mosquitoes of the prairie feed extensively on man, horse, cow, and bird. The degree to which the species feed upon a particular host appears to be determined largely by the availability of that host. The number of individual specimens reacting to more than one antiserum is high, on the average one out of three belonging in this group. In spite of the multiple feeding habits of the *Aedes* species and their relatively great abundance, they do not appear to play a major role in the transmission of western equine encephalomyelitis.

During the summer of 1941 the province of Saskatchewan experienced a great outbreak of human encephalomyelitis. Although extensive outbreaks of equine encephalomyelitis preceded it in 1935, 1937, and 1938, there were comparatively few cases in man prior to 1941. In that year the number of diagnosed human cases reached 543, with 44 deaths. The greatest number of cases, i.e., 72.9%, occurred during the last three weeks of August, following an excessive outbreak of mosquitoes during the latter part of July and early August. Although cases of the disease were widely distributed over the province, the greatest incidence was found in an area encompassing Regina, Moose Jaw, and Weyburn.

Owing to the fact that on epidemiological grounds mosquitoes here and elsewhere were seriously suspected as being the vectors of the disease, a study of the species of the Regina district was undertaken in 1942 and continued through 1943 and 1944. During the first two seasons the general ecology of the species was stressed (12), while in the last summer emphasis was placed on feeding habits as revealed by field observations, and by means of the precipitin test. It was felt especially desirable to determine to what extent individual mosquitoes feed on more than one host, for, barring hereditary transmission, multiple feeding by the vector is a requisite to an epidemic of an insect-borne disease.

The precipitin reaction has been frequently used in studies connected with the feeding habits of insects after its original suggestion by Uhlenhuth, Weidanz, and Angeloff (13).

In view of the fact that a number of recent workers (1, 3, 4, 5, 11) had reported favourably on the use of this method it was decided to apply it in the investigation of the blood meal of mosquitoes.

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*This investigation is part of a co-operative survey of encephalomyelitis in Saskatchewan by the Provincial Department of Public Health, the Federal Department of Agriculture, and the Departments of Veterinary Science and of Biology of the University of Saskatchewan.*

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## Laboratory Methods

*The Preparation of Antiserum*

In view of the fact that Saskatchewan is mainly an agricultural province, it was considered that mosquitoes would feed almost entirely on man and the domestic animals. Antisera were therefore prepared against human, equine, bovine, and avian serum. Human serum was obtained from the blood samples submitted for luetic serological examination, equine was purchased, while bovine and chicken blood were secured from the abattoir. Chicken serum was used as the representative of the avian group and in all cases filtered pooled sera were used.

The method of preparing the antiserum was essentially that given by Bull and King (3).

Fully grown, healthy, male rabbits were selected, and these were given a series of four intravenous injections of 5 cc. of serum on alternate days. A trial bleeding was made 10 days after the last injection, and the titre established. Titres in all cases except one were higher than 1:6400. The rabbits were starved for 18 hr., and then bled by cardiac puncture, about 30 cc. being obtained. The animals were bled again 48 hr. later. The rabbits were given a rest of one week, and the course of injection repeated.

In order to avoid anaphylactic reactions in the second series of injections, the rabbits were first given 1 cc. of serum subcutaneously. The following morning 1 cc. of serum diluted with 4 cc. of normal saline was given intravenously. In the afternoon 5 cc. of serum diluted with an equal amount of saline was given intravenously. This last injection was repeated three times at two day intervals. In spite of these precautions, it was found that rabbits that were injected with human serum suffered considerable shock during this second series of injections, although death did not occur. This response was not observed in the case of those injected with equine, bovine, and avian serum. Cardiac bleeding was done as before, but on the second bleeding the rabbits were killed and bled out aseptically.

The blood was allowed to clot and the serum was centrifuged. Fifty per cent of neutral glycerine was added as a preservative. This method was adopted after some experimentation with other methods of preserving the serum. 'Loading' small test-tubes was easier with a solution of greater density, and 'ring tests' were easier to read and not as easily dispersed as those of antisera preserved in other ways. All antisera were tested for absence of cross reactions before use.

*The Collection and Preparation of Specimens*

Gorged specimens of mosquitoes were identified in the field and the blood meal was pressed out on a good quality of analytical filter paper. Each droplet was numbered with a key number representing the species, and with a specimen number. A corresponding data sheet accompanied each set of specimens. A number of specimens in which the final feeding was known to the collector was submitted.

The filter paper was cut as close to the edge of the blood drop as possible and this small disk was placed in a ⅜ in. test-tube, which was then corked. Until the time of examination these were kept dry. When a series was to be examined, 0.5 cc. of saline was added to the disk of filter paper giving approximately a 1 : 50 dilution. The tubes were re-corked and placed in the refrigerator overnight. They were then ready for examination on the following morning. Experiments proved that this overnight extraction gives clearer reactions than extraction for a shorter time at room temperature, while a longer period of extraction gives cloudy and unsatisfactory results. Positive and negative controls were set up with each group of specimens.

*Effect of Time*

Table I indicates the readings obtained from specimens that were killed at various intervals after known human feedings. There was no observation or control of previous feedings. It appears that up to 18 hr. there is little difference in sensitivity or specificity of the reaction as far as can be judged from the small number of cases examined. For this reason also it is not possible to state whether there were more negative reactions or missed reactions as longer holding periods were used.

TABLE I

RESULTS OBTAINED FROM MOSQUITOES KILLED AT VARIOUS TIME INTERVALS AFTER KNOWN HUMAN FEEDINGS

| Interval between feeding and killing, hr. | Simultaneous reactions | | | | Multiple reactions in order of appearance | | | | Other reactions | | | No reactions | Total specimens |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *H* | *HE* | *HB* | *HA* | *HE* | *EH* | *BH* | *EAH* | *E* | *B* | *A* | | |
| 1–4 | — | — | 2 | 1 | 1 | 2 | 1 | — | — | — | — | — | 7 |
| 5–9 | 21 | 5 | 2 | 3 | 1 | 2 | — | 1 | 3 | 2 | 1 | 2 | 43 |
| 10–14 | 14 | — | — | — | — | — | — | — | 1 | — | — | 2 | 17 |
| 15–18 | 8 | 1 | — | — | 3 | — | — | — | — | — | — | 1 | 13 |
| Total | 43 | 6 | 4 | 4 | 5 | 4 | 1 | 1 | 4 | 2 | 1 | 5 | 80 |

NOTE: *H = human; E = equine; B = bovine; A = avian.*

In the series of mosquitoes that were known to have fed on the collector, it is interesting that of the 80 specimens examined, 68 showed human reaction, five showed no reaction, and seven showed reaction other than human. Of those giving human reaction, 25 gave at least one other reaction, and of these only five showed the human first, while 14 occurred simultaneously, and in six cases another reaction occurred prior to the appearance of the human reaction.

A number of specimens were collected during the summer of 1943 and were not examined until June 1944 and during this time the blood meal had been stored as dried droplets on filter paper. There was apparently a higher proportion of negative tests but otherwise the results appeared to be quite satisfactory and the readings quite clear.

## Method of Performing and Reading the Test

After some preliminary investigation the following procedures were adopted.

*Equipment*

(*a*) Small test-tubes, 4 mm. × 40 mm. (four tubes for each specimen). Cleaning these small tubes proved to be a time consuming task. It was necessary to load each one with cleaning fluid, and to add the distilled water for rinsing in the same way.

(*b*) Small test-tube racks to fit the above. These were made from masonite in our own laboratory. Each had two rows of 10 holes, which were staggered to facilitate reading. Thus two racks were required for each set of 10 specimens.

(*c*) Capillary pipettes with long tips.

*Method*

Tubes of Row No. 1 were loaded with one drop of glycerinated human antiserum, using a capillary pipette to deliver the serum to the bottom of the tube. The second row of tubes was similarly loaded with equine antiserum, the third with bovine, and the fourth with avian.

The first tubes of antiserum in each row were stratified with one drop of extract from the first specimen, the second tubes with the second specimen and so on. It was found that, allowing the necessary time for reading between incubations, 40 specimens were all that could be handled satisfactorily at one time.

*Reading*

The tests were incubated at 37° C. for 10 min., when they were removed and the first reading was made. Readings were made before a slit lamp.

The racks were returned to the incubator, and further readings were made at 20, 30, 45, and 60 min. intervals. No fresh reactions were observed in this series after the 30 min. reading and, at the end of 60 min., most of the reactions had faded out.

## Species Studied

The species studied in this survey are those most abundant in the district (12). Some of these, namely, *Aedes vexans* (Meigen), *A. dorsalis* (Meigen), and *A. nigromaculis* (Ludlow) have been shown elsewhere to be capable of transmitting the virus of encephalomyelitis to laboratory animals (6). *Culex tarsalis* Coquillett and *Culiseta inornata* (Williston) are not represented in the study because of the small numbers available during the 1944 season. This is regrettable because both species have repeatedly been shown by Hammon to harbour the virus in nature (7, 9), and also readily to transmit it to laboratory animals (7, 10). Moreover, the work of Hammon *et al.* lends strong support to the view that *Culex tarsalis* is the main vector of the disease (8).

A survey made in the Regina district in late August, 1941, showed that the species represented in the great mosquito outbreak that preceded the ence-

phalomyelitis epidemic were *Aedes nigromaculis*, *A. spencerii* (Theobald), *A. dorsalis*, *A. vexans*, and *A. campestris* (Dyar and Knab). Larvae of *Culex tarsalis* and *Culiseta inornata* were exceptionally abundant in late August of that year, suggesting that both species were well represented in the earlier outbreak.

## Results and Interpretation

The results of the precipitin tests indicate that the *Aedes* species of the prairie feed extensively on man, horse, cow, and bird (Tables II and III). In spite of the fact that the numbers dealt with in this paper are small, it was decided to express them in the form of percentages. Although these results in general are in agreement with field observations on the feeding habits of the species, the number reacting to avian antiserum was felt to be surprisingly high (Table III). However, owing to the absence of species specificity in the case of avian antiserum, these specimens may have fed not only on chickens, but also on other domestic fowl and on wild birds. The number of specimens not reacting to any of the four antisera is low, suggesting that only on rare occasions is the blood meal obtained from a host other than one of the four mentioned above. *Aedes flavescens* (Mueller) is an exception. When collected in a habitat with few or no domestic animals in the neighbourhood (Table II (*a* and *c*)), almost one-fourth are negative, suggesting that some other animal, such as a gopher, may be the host. Of the species studied only *A. flavescens* will not readily migrate for a long distance from its breeding habitat in search of food. None of the species studied show a decided host preference. The degree to which the species feed upon a particular host appears to be determined largely by the availability of that host. Thus at the outskirts of the city the percentage of those that feed on man is higher than in the country. Also, mosquitoes collected near an Indian encampment at the outskirts of the city with several hundred horses grazing in the pasture, react in great numbers to horse and human antisera.

The number of individual specimens reacting to more than one antiserum is high, on the average one out of three belonging in this group. Frequently no mention of double positives is made in the literature. When they have been observed, it has been the custom, for statistical purposes, to call them negative, or to assign half value to each, or to call the first and stronger reaction positive (2), ignoring the other. However Badzhiev (1), in his work on *Anopheles* mosquitoes, records frequent multiple positives (almost 30% not counting those giving cross species reactions) and assigns equal value to all.

In the present work double positives were frequently observed and in most cases neither one nor the other reaction was more prominent. The difference, when observed, was merely one of time. However, we feel that the difference in the time of the appearance of the precipitate is dependent upon the concentration of the antigen at the interface between antiserum and extract. A high concentration at the interface will result in an immediate reaction, while in the case of a low concentration the reaction has to await the diffusion of antigen into the region of the interface. But this requires time.

## TABLE II

FEEDING HABITS OF SASKATCHEWAN MOSQUITOES

| Source of mosquitoes | Species of mosquitoes | Percentage of blood smears reacting to specific antisera | | | | | | | | | | | | | | % Neg. | No. tested |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *H* | *HE* | *HB* | *HA* | *E* | *EB* | *EA* | *B* | *BA* | *A* | *HEB* | *HEA* | *HBA* | *EBA* | | |
| (*a*) Collected over vegetation in pasture. No domestic animals present at time | *Aedes spencerii* | 8.0 | 8.9 | 2.8 | 2.8 | 21.2 | 14.1 | 7.0 | 16.0 | 6.1 | 8.5 | 0.9 | 0.5 | | 1.4 | 1.8 | 213 |
| | *A. flavescens* | 15.6 | 20.0 | 2.2 | 6.7 | 6.7 | 2.2 | 11.1 | 4.4 | 2.2 | 15.6 | 2.2 | | | | 11.1 | 45 |
| | *A. vexans* | x | | | | x | | | | | | | | | | | 2 |
| | *A. nigromaculis* | 21.3 | | 7.1 | 7.1 | 7.1 | 14.4 | | 14.4 | | 14.4 | | 7.1 | | | 7.1 | 14 |
| | *A. dorsalis* | | | | | | | | | | | | | | | | 0 |
| (*b*) Collected over vegetation in pasture near farm yard. Domestic animals near | *A. spencerii* | 11.8 | 8.2 | 2.7 | 8.2 | 25.4 | 4.5 | 8.2 | 6.4 | 6.4 | 12.7 | | | 1.0 | | 4.5 | 110 |
| | *A. flavescens* | 8.8 | 7.5 | 2.5 | 2.5 | 25.0 | 15.0 | 15.0 | 10.0 | 3.7 | 8.8 | | | | 1.2 | | 80 |
| | *A. vexans* | 12.4 | 4.2 | 4.2 | 4.2 | 14.5 | 8.3 | 4.2 | 16.7 | 8.4 | 14.5 | | | 2.1 | 4.2 | 2.1 | 48 |
| | *A. nigromaculis* | 24.0 | 12.0 | 2.4 | 5.2 | 21.0 | 6.0 | 6.0 | 9.8 | 1.6 | 5.2 | | 0.8 | 0.8 | | 5.2 | 133 |
| | *A. dorsalis* | | 9.1 | | | 36.3 | 9.1 | | 36.4 | | 9.1 | | | | | | 11 |
| (*c*) Collected over vegetation near outskirts of city. No domestic animals nearby | *A. spencerii* | 25.5 | 4.2 | 4.2 | 12.7 | 18.3 | 2.8 | 5.6 | 8.5 | 2.8 | 7.0 | 1.4 | | 1.4 | 1.4 | 4.2 | 71 |
| | *A. flavescens* | 17.6 | 12.3 | 1.7 | 3.5 | 14.0 | 5.3 | 3.5 | 5.3 | 1.7 | 10.5 | | | 1.7 | | 22.9 | 57 |
| | *A. vexans* | 18.8 | | | | 18.7 | 6.3 | 6.3 | 15.6 | 3.1 | 21.8 | | | 3.1 | | 6.3 | 32 |
| | *A. nigromaculis* | 11.1 | | | 11.1 | 11.1 | 22.2 | | 11.1 | | 22.3 | | | | | 11.1 | 9 |
| | *A. dorsalis* | 21.1 | 12.1 | 2.3 | 5.3 | 13.6 | 2.3 | 2.3 | 10.6 | 2.3 | 25.0 | | | 0.8 | | 2.3 | 132 |
| (*d*) Collected over vegetation near outskirts of city beside Indian encampment | *A. spencerii* | 17.3 | 15.9 | 1.4 | 4.2 | 33.2 | 2.1 | 4.9 | 6.3 | 1.4 | 6.3 | | 0.7 | | | 6.3 | 144 |
| | *A. flavescens* | 12.1 | 20.6 | 8.6 | | 22.4 | 6.9 | | 6.9 | | 12.1 | 1.8 | | | | 8.6 | 58 |
| | *A. vexans* | 28.8 | 5.3 | 2.7 | | 28.8 | 7.9 | | 7.9 | 2.7 | 7.9 | | | | | 8.0 | 38 |
| | *A. nigromaculis* | 30.0 | 10.0 | | | 20.0 | 10.0 | 10.0 | 20.0 | | | | | | | | 10 |
| | *A. dorsalis* | 21.8 | 39.3 | 4.3 | 4.3 | 8.6 | | 4.3 | 13.1 | | 4.3 | | | | | | 23 |
| Total | | | | | | | | | | | | | | | | | 1230 |

NOTE: *H = human; E = equine; B = bovine; A = avian; x represents one specimen.*

TABLE III

FEEDING HABITS OF SASKATCHEWAN MOSQUITOES

| Source of mosquitoes | Species of mosquitoes | Percentage of blood smears reacting to specific antisera | | | | % Neg., | No. tested |
|---|---|---|---|---|---|---|---|
| | | Percentage of total reacting to human antiserum | Percentage of total reacting to equine antiserum | Percentage of total reacting to bovine antiserum | Percentage of total reacting to avian antiserum | | |
| (a) Collected over vegetation in pasture. No domestic animals present at time | *A. spencerii* | 23.9 | 54.0 | 41.3 | 26.3 | 1.8 | 213 |
| | *A. flavescens* | 46.7 | 42.2 | 13.3 | 35.6 | 11.1 | 45 |
| | *A. vexans* | x | x | | | | 2 |
| | *A. nigromaculis* | 42.9 | 28.6 | 35.7 | 28.6 | 7.2 | 14 |
| | *A. dorsalis* | | | | | | 0 |
| (b) Collected over vegetation in pasture near farm yard. Domestic animals near | *A. spencerii* | 31.9 | 46.3 | 21.0 | 36.5 | 4.5 | 110 |
| | *A. flavescens* | 21.3 | 63.7 | 32.4 | 31.2 | | 80 |
| | *A. vexans* | 27.1 | 35.4 | 43.9 | 37.6 | 2.1 | 48 |
| | *A. nigromaculis* | 45.1 | 45.9 | 20.3 | 19.5 | 5.3 | 133 |
| | *A. dorsalis* | 9.1 | 54.5 | 45.5 | 9.1 | | 11 |
| (c) Collected over vegetation near outskirts of city. No domestic animals nearby | *A. spencerii* | 58.5 | 33.7 | 22.5 | 30.9 | 4.2 | 71 |
| | *A. flavescens* | 36.8 | 35.1 | 15.7 | 20.9 | 22.9 | 57 |
| | *A. vexans* | 21.9 | 31.3 | 28.1 | 34.3 | 6.3 | 32 |
| | *A. nigromaculis* | 22.2 | 33.3 | 33.3 | 33.4 | 11.1 | 9 |
| | *A. dorsalis* | 41.6 | 30.3 | 18.3 | 35.7 | 2.3 | 132 |
| (d) Collected over vegetation near outskirts of city beside Indian encampment | *A. spencerii* | 39.5 | 56.8 | 11.2 | 17.5 | 6.3 | 144 |
| | *A. flavescens* | 43.1 | 51.7 | 24.2 | 12.1 | 8.6 | 58 |
| | *A. vexans* | 36.8 | 42.0 | 21.2 | 10.6 | 8.0 | 38 |
| | *A. nigromaculis* | 40.0 | 50.0 | 30.0 | 10.0 | | 10 |
| | *A. dorsalis* | 69.7 | 52.2 | 17.4 | 12.9 | | 23 |
| Total | | | | | | | 1230 |

To accept, then, the first reaction and ignore the second, appears completely to invalidate the use of the precipitin test. It should also be pointed out that on a number of occasions two reactions occurred *simultaneously*. One might well ask, then, which was to be considered positive. It should be stressed also that none of these double positive tests belonged to recognized cross-species reactions.

We interpret our results in the following manner. In the case of two reactions occurring simultaneously, the concentration of antigen at the interface was the same. The condition could obtain if blood in equal amounts were taken from two hosts with little time between the feedings, or if the first feeding was complete, followed after a considerable interval by a partial second feeding. Similarly, in the case of two reactions not occurring simultaneously, the first reaction may indicate the last feeding, or merely a greater blood meal. The tests, therefore, disclose the host or hosts, but do not reveal the order of feeding, nor the relative amounts of blood taken at each feeding.

If our readings reflect a true picture, then the *Aedes* species of the prairie cannot be looked upon as important vectors of encephalomyelitis. During the past few summers *Aedes* species have on occasion been very abundant in certain localities and on the basis of the precipitin test they fed extensively on more than one host. Yet the expected outbreak of the disease did not occur unless *Culiseta inornata* and *Culex tarsalis* were present. This indirect evidence incriminating the last two species as the main vectors of the disease is in complete agreement with the findings in California and elsewhere.

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# THE REDUCTION OF HYDROGEN PEROXIDE BY FIBRIN[1]

By L. B. Jaques[2] and H. J. Bell[3]

## Abstract

It has previously been reported by Jaques that fibrinogen and fibrin possess marked reducing powers. In a further investigation of this property, the reduction of hydrogen peroxide by these proteins has been studied. Commercial fibrin sometimes contains large amounts of catalase. Methionine in phosphate buffer reduces hydrogen peroxide at pH 6.6 and temperature of 25° C. This reduction is increased by copper and molybdate. The reduction of hydrogen peroxide by fibrinogen and fibrin free of catalase is due to the methionine residue.

Jaques (3) has previously reported that fibrinogen and fibrin show marked reducing properties at pH 6.6 and 25° C. While the reduction of iodine was traced to the aromatic residues (tyrosine and tryptophane), the reduction of hydrogen peroxide was not due to these amino acid groups. Cysteine was not a factor since these proteins fail to give a direct nitroprusside reaction, even after denaturation. Toennies and Callan (8) have shown that methionine is a stronger reducing agent for hydrogen peroxide than cysteine and we have investigated the contribution of methionine to the reaction previously reported. It appears likely that methionine is responsible for the effect observed. A further factor has been demonstrated, namely, that some commercial preparations of fibrin contain large amounts of catalase.

## Materials and Methods

These were the same as in the previous study, the fibrinogen being prepared from horse plasma by a method similar to that of Florkin. In the preparation chiefly used, the horse plasma was subjected to a preliminary treatment with tricalcium phosphate. Commercial fibrin was obtained from British Drug Houses, Ltd. and Difco Laboratories, Inc. The protein and buffer, water, hydrogen peroxide, etc. were placed in glass-stoppered flasks in a constant temperature bath at 25° C. The reaction was stopped by adding potassium iodide and 4*N* sulphuric acid and the residual hydrogen peroxide determined by titration of the equivalent iodine with sodium thiosulphate. Measurements were made with the Beckman glass electrode.

## Results and Discussion

### *The Catalase Activity of Fibrin*

When a commercial preparation of Difco fibrin was tested for reducing power, it was found that it rapidly destroyed large quantities of hydrogen peroxide (Table I). Thus 100 mgm. of fibrin decomposed 10.5 ml. of *N*/10

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hydrogen peroxide in one hour and 54 ml. in 16 hr. This is many times the reducing power of fibrin previously studied and since it is equivalent to a reduction of 184 mgm. of hydrogen peroxide per 100 mgm. of protein, it must be due to a catalytic decomposition of the hydrogen peroxide and not to a simple reaction with a protein group. This was confirmed by collection of the oxygen in a spirometer.

TABLE I

THE DESTRUCTION OF HYDROGEN PEROXIDE BY DIFCO FIBRIN

| Time | $N/10$ $H_2O_2$ destroyed, ml.* |
|---|---|
| 11½ min. | 2.18 |
| 37 " | 4.88 |
| 60 " | 10.49 |
| 91 " | 11.57 |
| 120 " | 15.62 |
| 16 hr. | 53.70 |
| 30 " | 54.66 |

NOTE: $H_2O_2 = 0.888$ *M.*
* *By 100 mgm. fibrin.*

Experiments were conducted to determine the nature of the catalytic agent. Heating the fibrin suspended in buffer for five minutes at 100° C. destroyed its reducing power, with the exception of a small amount of residual reduction. The effect of incubation at 40° C. at various pH levels for one hour was studied, the reducing activity being tested at pH 6.6. Complete loss of activity occurred at pH 2.2 and 4.0 but no loss at 6.0 and some loss at pH 8.0. The addition of $M/400$ cyanide resulted in about 50% inhibition. It is probable, therefore, that the catalytic reduction of hydrogen peroxide is due to the presence of catalase in the fibrin preparation. It was found that the catalase is not always distributed uniformly through the sample, causing practical difficulties in studying this activity. Fibrin from British Drug Houses, Ltd. showed some catalase activity, although much less than the Difco fibrin. Thus, 100 mgm. decomposed only 0.8 ml. of $N/10$ hydrogen peroxide after 30 hr.

The presence of catalase activity in fibrin was first reported by Thenard in 1818 (cf. 2). It is of interest that an enzyme as unstable as catalase, when adsorbed on fibrin, survives the drastic procedures involved in the commercial preparation of the fibrin. The catalase probably comes from the red cells since the Difco fibrin, containing large amounts of the enzyme, is prepared by defibrinating whole blood.

*The Non-catalase Reducing Power of Fibrin*

After destruction of the catalase activity of fibrin, there still remains the reducing activity previously reported. It was found that this activity is

not destroyed by heat or by hydrolysis with hydrochloric acid, sulphuric acid, baryta, or trypsin. Qualitative tests for ferrous and ferric iron on the ash from fibrin were completely negative. Samples of fibrin and of fibrin oxidized with hydrogen peroxide under the conditions used were subjected to various colour tests. No difference between the oxidized and unoxidized protein was observed for the following reactions—biuret, Molisch, xanthoproteic, Millon, Adamkiewcz, Ehrlich, Sullivan and Hess, Sakaguchi, ninhydrin, and the lead acetate test for sulphur. However, the McCarthy and Sullivan test for methionine, which was strongly positive before oxidation, was completely negative afterwards. Hence the reduction must be, at least in part, due to the oxidation of methionine.

Toennies and Callan have studied the oxidation of methionine by hydrogen peroxide in acid media. They found that the reaction is accelerated by increasing the acidity to above pH 1.0 and that the oxidation of methionine was confined to the sulphoxide stage, but if molybdate or perchloric acid were added, the oxidation proceeded to the sulphone. In a preliminary experiment we found that methionine reduced hydrogen peroxide under the conditions used. This was further studied as shown in Table II.

TABLE II

FACTORS INFLUENCING OXIDATION OF METHIONINE BY $H_2O_2$

| Buffer, etc. | pH | $H_2O_2$ reduced, ml. of $N/200$ |
|---|---|---|
| HCl | 0.90 | 1.037 |
| HCl | 2.35 | 0.859 |
| Acetate | 4.51 | 0.793 |
| Acetate | 4.73 | 0.568 |
| Acetate | 5.03 | 0.453 |
| Acetate | 5.62 | 0.130 |
| Acetate + phosphate | 5.47 | 0.617 |
| Acetate + molybdate | 4.93 | 1.052 |

NOTE: *Methionine* $2.68 \times 10^{-3}$ *M.*
*$H_2O_2$* *0.0365 M.*
*Molybdate* $4 \times 10^{-4}$ *M ammonium molybdate.*
*Phosphate* *M/24 potassium phosphate, pH 6.6.*
*Temperature* *25° C.*
*Reaction time* *41 hr.*

As shown by Toennies and Callan, methionine reduces hydrogen peroxide in strongly acid solutions. However, reduction of hydrogen peroxide still occurs above pH 4, although it falls off rapidly between pH 4.5 and 5.6. Both phosphate and molybdate ions markedly increase the reduction. Hence it appears evident that while the reduction by methionine alone is negligible above pH 6.0, yet the presence of phosphate or molybdate will result in the measurable reduction found in the preliminary experiment. The reaction in phosphate at 25° C. and pH 6.6 reaches an equilibrium in 15 to 24 hr. with

50% reduction being reached after six hours. At equilibrium a linear relation was obtained between methionine concentration and hydrogen peroxide reduced.

Toennies and Callan reported that copper had no effect on the reduction of hydrogen peroxide by methionine. Since copper catalyses the reduction of hydrogen peroxide by cysteine (5, 7), this might be used to distinguish the reduction by cysteine and methionine. Table III shows the number of ml. of $N/200$ hydrogen peroxide reduced by 1 mgm. methionine in the presence of various reagents. Phosphate, copper, and molybdate markedly increase the

TABLE III

REDUCTION OF HYDROGEN PEROXIDE BY METHIONINE, FIBRIN, AND EGG ALBUMIN

| Solvent | $N/200$ $H_2O_2$ reduced, ml. | | |
|---|---|---|---|
| | Methionine, 1 mgm. | Fibrin, 8.33 mgm. | Egg albumin, 1.66 mgm. |
| Water | 0.35 | 0.11 | 0.14 |
| Phosphate buffer | 0.59 | 0.23 | 0.25 |
| Copper + water | 1.72 | 3.17 | 2.40 |
| Copper + phosphate | 2.42 | 3.89 | 1.93 |
| Molybdate + water | 3.92 | 2.72 | 0.41 |
| Molybdate + copper + water | 4.73 | 4.16 | 3.15 |

*$H_2O_2$* *0.043 M.*
*Copper* *$1.3 \times 10^{-3}$ M copper sulphate.*
*Molybdate* *$4.2 \times 10^{-4}$ M ammonium molybdate.*
*Phosphate* *M/24 potassium phosphate, pH 6.6.*
*Reaction time* *= 41 hr.*

reduction by methionine, the copper and molybdate being particularly effective. The effects of phosphate and copper and of molybdate and copper are additive. The presence of copper caused some difficulty in the titrations, however. As in the other experiments, the values reported show the increased reduction compared to suitable controls with methionine or protein omitted. Reduction in the control flasks was very small under the conditions used. The difference in the effect of copper from that reported by Toennies and Callan is presumably due to the quite different pH level used. Also shown are the results obtained with fibrin and egg albumin. The reduction by both proteins is increased by phosphate, copper, and molybdate. Egg albumin shows a marked increase with copper and a relatively smaller increase with molybdate, while the increased reduction with copper is decreased by phosphate. The results with fibrin resemble those with methionine, although the increase in the presence of copper is greater than would be expected on this basis. Toennies and Callan have previously suggested that the increased reduction of hydrogen peroxide by proteins in the presence of copper and molybdate is due in part to components other than methionine and cysteine.

In order to assess the contribution of methionine to the reduction of hydrogen peroxide by fibrin and fibrinogen, the methionine content of fibrinogen and fibrin was determined by the method of Kolb and Toennies (4). The values found were 2.95% in fibrin and 2.77% in horse fibrinogen. Brand, Kassell, and Saidel (1) report values for methionine, obtained by other methods, of 2.62 to 2.72% in fibrin and 2.52% in fibrinogen. The total reduction by the protein, the reduction after heating for five minutes at 100° C., and the reduction by the amount of methionine present was then determined for 40 hr. in phosphate buffer at pH 6.6. The reductions in ml. of $N/200$ hydrogen peroxide per 10 mgm. of protein were for fibrin, total reduction, 1.565; residual reduction after heating, 0.236; reduction by equivalent methionine, 0.252. No loss of reduction occurred after heating the fibrinogen. Reduction by fibrinogen was 0.273 and by the methionine, 0.251. The reduction by fibrin was equivalent to the reduction by the catalase plus methionine present, while the reduction by fibrinogen and the methionine it contained were the same, so it appears likely that these two factors are alone responsible for the reduction by these proteins. No consistent difference in reducing power between fibrinogen and fibrin has been found. It appears probable that a trace of catalase in the sample of fibrin previously used was responsible for the increased reduction by fibrin reported.

It is evident that the reduction of hydrogen peroxide by methionine under relatively mild conditions is a property also of methionine present in protein and may be of considerable significance in considering the properties of proteins. However, it does not appear to be of value for the determination of methionine in protein unless the protein is first hydrolysed, as in the method of Kolb and Toennies.

After we had completed this study, Perlman and Lipmann (6) published the results of a comprehensive study of the catalase in serum and plasma. They reported that slight haemolysis increased the serum catalase in proportion to the amount of haemoglobin released. However, normal sera contained approximately uniform amounts of catalase.

## Acknowledgments

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# THE SEROLOGICAL RELATIONSHIPS OF THE RICKETTSIAE OF EPIDEMIC AND MURINE TYPHUS[1]

By James Craigie[2], Dennis W. Watson[3], Eina M. Clark[4], and M. Elizabeth Malcomson[5]

## Abstract

The complement-fixing antibodies present in epidemic and murine typhus immune sera can be differentiated by quantitative absorption tests. The neutralizing antibodies that participate in the Giroud reaction can be differentiated by quantitative inhibition tests.

Epidemic and murine typhus immune sera contain two kinds of complement-fixing antibodies and also two kinds of neutralizing antibodies. The antigens that react with these antibodies differ in specificity and thermal resistance. Cross reactions between epidemic and murine rickettsiae are due to the presence of similar heat stable antigens in the two types. Type specific sera may be obtained by absorbing immune serum with either (*a*) rickettsiae of heterologous type or (*b*) heated rickettsiae of homologous type. The specific antibodies react only with the heat labile antigens of the homologous type of rickettsiae.

Mice may be actively immunized against the toxic factors of murine and epidemic rickettsiae. The immunity produced by small doses of vaccine is type specific and dependent on the presence of heat labile antigen in the vaccine.

In the absence of data obtained from reciprocal cross absorption tests, any one of the following three hypotheses might be used to explain the serological and immunological relationships of murine and epidemic typhus rickettsiae.

1. The two types of rickettsiae share the same antigens in different proportions, i.e., each type contains a small amount of the antigen that characterizes the heterologous type.
2. The epidemic type is the murine type that has acquired additional antigens.
3. Epidemic and murine types of rickettsiae possess different type specific antigens and their observed cross immunological reactions are referable to an additional antigen common to both types.

If major importance is attached to the cross immunity that occurs between epidemic and murine typhus, and if Zinsser's (16) suggestion of transformation of the murine type to the classic epidemic type by repeated louse–man passage is accepted, it might seem to be unnecessary to postulate type specific antigens. Either the first or the second hypothesis might explain the cross

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immunity that develops after infection with one of the two types. The partial cross immunity of guinea-pigs vaccinated with killed rickettsiae could be explained by the presence of a relatively small quantity of heterologous antigen, but is somewhat more difficult to explain on the basis of the second hypothesis unless it is postulated that the additional epidemic antigen prevents adequate response to the murine component. Intermediate strains of typhus could be regarded either as having a more equal ratio of epidemic and murine antigen (first hypothesis), or as having less epidemic antigen that is found in typical epidemic strains (second hypothesis).

Zinsser and Castaneda (17) observed that although cross agglutination occurs between epidemic and murine rickettsiae, immune sera react to a higher titre with the homologous type. Van Rooyen and Bearcroft (15) have recently reported similar results obtained with convalescent sera from cases of epidemic and murine typhus. In the case of the complement fixation test, marked cross reactions may occur*; but Plotz (13) has reported on the use of rickettsial antigens that yield more specific results. Plotz carried out absorption tests on specimens of serum from cases of Brill's disease. Murine rickettsiae absorbed only the murine antibody, but epidemic rickettsiae removed antibodies for both murine and epidemic rickettsiae respectively. Plotz suggested that the epidemic antigen may be more complex than the endemic antigen. This view would be in keeping with the second hypothesis already mentioned.

Neither the first nor the second hypothesis takes into account the heat labile and heat stable antigens of rickettsiae. The most recent discussion of these antigens is that presented by Felix (7). Felix draws an analogy with the Vi and O antigens of *Bacterium typhosum* but does not enlarge on the differences between the rickettsiae of murine and epidemic typhus. Actually, the heat lability of certain rickettsial antigens provides one of the most important clues to differences in antigenic structure. The specific agglutinogens of epidemic and murine strains are labile at 56° C. It was this that led the senior author to use these agglutinogens as a guide in the development of ether processing methods that aimed at the retention of the maximum amount of labile antigen in the rickettsiae. More recently, a direct mouse test (4, 6) was investigated and adopted as an antigenic test for typhus vaccine prepared by the Cox method of culture and processed at pH 7.0 by the method described in Memo. No. 5 (5). It has been found that the protection conferred by this type of vaccine is highly specific and that the immunizing antigen is, like the specific agglutinogen, labile at 56° C. Such findings suggested that the third hypothesis, presupposing two type specific antigens, was more probably correct, but other observations indicated some further degree of antigenic complexity. For example, complement fixation tests failed to show a commensurate destruction of antigen in vaccines heated at 56° or 60° C. When the serological responses of guinea-pigs to unheated and heated vaccine were compared, there was no evidence that heating impaired the capacity of the vaccine

* *Unpublished observations.*

to stimulate complement-fixing antibodies or neutralizing antibodies (Giroud test).

It therefore became necessary to resort to complete reciprocal cross absorption tests*, utilizing the fact that some of the rickettsial antigens are heat labile. The results of a number of these tests are presented in the sections that follow**.

## I. The Complement-fixing Antigens of Epidemic and Murine Strains

### Materials

*Strains of Rickettsiae*

The following strains were used: (*a*) Breinl epidemic; (*b*) Madrid epidemic; and (*c*) Castaneda murine.

*Guinea-pig Sera*

These were obtained from guinea-pigs infected with yolk sac cultures. The animals were bled 14 to 21 days after the onset of fever. Sera from several guinea-pigs inoculated with the same strain were pooled and inactivated at 56° C. None of these sera contained demonstrable mounts of yolk antibody.

*Antigens*

(1) Soluble antigens. Ether processed vaccine stocks*** were centrifuged in an angle head at 4000 r.p.m. for 45 min. The supernatants, which contained the soluble antigens, were used in dilutions determined by preliminary titrations. In their undiluted state they contained approximately 0.02% formalin and 1 in 10,000 merthiolate.

(2) Washed rickettsiae. The rickettsiae that had been separated from the soluble antigen by centrifugation were suspended in saline containing 0.5% formalin and subjected to further purification with ether. The suspensions were used for the titration of complement-fixing antibody in dilutions determined by preliminary titrations. The dense suspensions used for absorbing the sera were 30 to 50 times more concentrated than this.

### Methods

*Complement Fixation Tests*

The method of Plotz and Wertman (14) was used with the reagents described.

*Absorption Tests*

The required amount of the absorbing suspension of rickettsiae was centrifuged in an angle centrifuge, the supernatant was decanted, and the remaining drops of fluid were removed with strips of sterile filter paper. The serum that was to be absorbed was intimately mixed with the deposit of rickettsiae in a dilution of 1 in 6.25. After being kept at room temperature for two hours,

** Since the experimental work described in this memorandum was undertaken, two reports (1, 11) containing data on the results of cross absorption tests have been received (see ensuing discussion).*

*** The complement fixation tests were undertaken by M.E.M., the modified Giroud tests by D.W.W., and the mouse tests by J.C. and E.M.C.*

**** Prepared at pH 7.0 from yolk sac cultures as described in Memo. No. 5 (5).*

during which period they were frequently agitated, the mixtures were stored at 4° C. overnight. On the following morning, the mixtures were centrifuged to remove the rickettsiae, and the sera were titrated for residual antibody. In each test, a similarly diluted control sample of serum was prepared and treated in the same way except that rickettsiae were not added. The chief relationships of the antigens and antibodies participating in the complement fixation reactions are shown in the following experiments. The first of these presents the results of cross absorption tests with the epidemic and murine strains, while the others provide information concerning the heat stability of the antigens involved in the complement fixation reactions.

## Quantitative Cross Absorption Tests with Epidemic and Murine Strains

In these tests, different absorbing doses of rickettsiae were used for each serum. The results, which are summarized in Table I, show that rickettsiae of heterologous type can effectively absorb an antibody to an antigen common to both types but are unable to remove an antibody that reacts specifically with the homologous type. The homologous type, on the other hand, absorbs both antibodies with equal facility. Figs. 1 and 2 present the results of the quantitative absorption tests in graphic form.

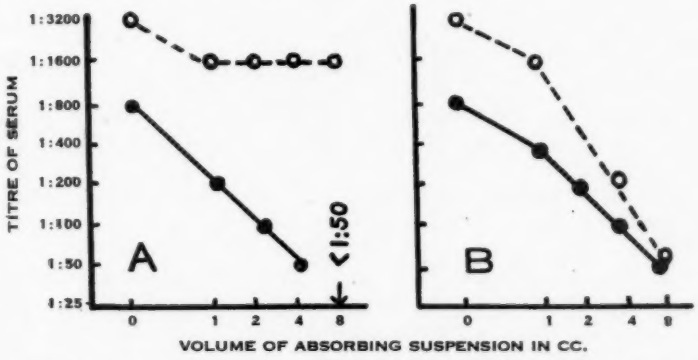


FIG. 1. *Quantitative absorption tests. A = Breinl convalescent serum absorbed with Breinl rickettsiae; B = Breinl convalescent serum absorbed with murine rickettsiae.* ● *Titre for Breinl antigen.* ○ *Titre for murine antigen.*

These tests (Table I) indicate that there are two type specific antigens and a common antigen and thus would seem to provide evidence against the view that the cross reactions between epidemic and murine strains result from the presence in these strains of two antigens, epidemic and murine, in different proportions. It may be noted that these tests also indicate that the specific complement-fixing antigens of the Breinl and Madrid strains are identical.

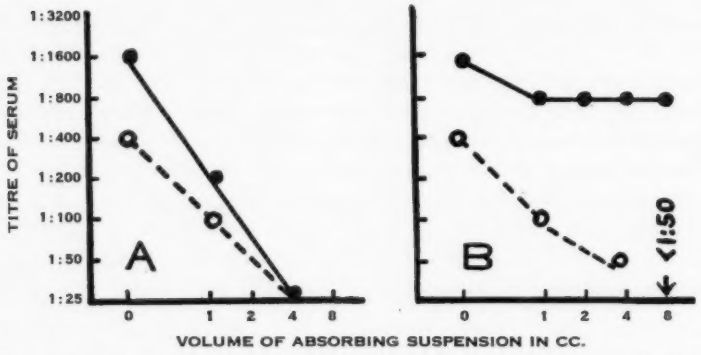


FIG. 2. *Quantitative absorption tests. A = murine convalescent serum absorbed with Breinl rickettsiae; B = murine convalescent serum absorbed with murine rickettsiae.* ● *Titre for Breinl antigen.* ○ *Titre for murine antigen.*

TABLE I

QUANTITATIVE CROSS ABSORPTION TESTS WITH EPIDEMIC AND MURINE TYPHUS RICKETTSIAE

| Serum | Date of testing | Treatment of serum | Complement-fixing titre of serum | | |
|---|---|---|---|---|---|
| | | | Breinl soluble antigen | Madrid soluble antigen | Murine soluble antigen |
| Breinl pooled convalescent guinea-pig serum | 7/7/43 | Not absorbed | 1 : 1600 | 1 : 1600 | 1 : 400 |
| | | Absorbed with Breinl 1 cc. | 1 : 200 | 1 : 200 | 1 : 100 |
| | | Absorbed with Breinl 2.5 cc. | <1 : 100 | — | <1 : 100 |
| | | Absorbed with Breinl 4 cc. | 1 : 25 | 1 : 25 | 1 : 25 |
| | | Absorbed with murine 1 cc. | 1 : 800 | — | 1 : 100 |
| | | Absorbed with murine 2.5 cc. | 1 : 800 | — | 1 : 100 |
| | | Absorbed with murine 4 cc. | 1 : 800 | 1 : 800 | 1 : 50 |
| | | Absorbed with murine 8 cc.* | 1 : 800 | — | <1 : 50 |
| Murine pooled convalescent guinea-pig serum | 7/7/43 | Not absorbed | 1 : 800 | 1 : 800 | 1 : 3200 |
| | | Absorbed with murine 1 cc. | 1 : 400 | — | 1 : 1600 |
| | | Absorbed with murine 2.5 cc. | 1 : 200 | — | — |
| | | Absorbed with murine 4 cc. | 1 : 100 | 1 : 200 | 1 : 200 |
| | | Absorbed with murine 8 cc. | 1 : 50 | — | 1 : 50 |
| | | Absorbed with Breinl 1 cc. | 1 : 200 | — | 1 : 1600 |
| | | Absorbed with Breinl 2.5 cc. | 1 : 100 | — | 1 : 1600 |
| | | Absorbed with Breinl 4 cc. | 1 : 50 | 1 : 100 | 1 : 1600 |
| | | Absorbed with Breinl 8 cc.* | 1 : 50 | — | 1 : 1600 |
| Madrid pooled convalescent guinea-pig serum | 10/8/43 | Not absorbed | 1 : 1600 | 1 : 3200 | 1 : 200 |
| | | Absorbed with Madrid 1 cc. | 1 : 100 | 1 : 100 | <1 : 50 |
| | | Absorbed with Madrid 8 cc. | <1 : 50 | <1 : 50 | <1 : 50 |
| | | Absorbed with Breinl 1 cc. | 1 : 800 | 1 : 800 | 1 : 100 |
| | | Absorbed with Breinl 8 cc. | <1 : 50 | 1 : 50 | <1 : 50 |
| | | Absorbed with murine 1 cc. | 1 : 1600 | 1 : 1600 | 1 : 100 |
| | | Absorbed with murine 4 cc. | 1 : 1600 | 1 : 1600 | 1 : 50 |
| | | Absorbed with murine 8 cc.* | 1 : 1600 | 1 : 1600 | <1 : 50 |

— *Not tested.*

* *Tests made 10/8/43.*

### The Thermostability of the Complement-fixing Antigens

Tests have been carried out with antigens heated for periods of one hour in order to determine the temperature at which the specific complement-fixing antigen loses its ability to react *in vitro*. These have indicated that the critical temperature of inactivation of the specific antigen lies between 56° and 65° C. and that the residual antigen is shared by the epidemic and murine types.

For example, Breinl convalescent serum that had been absorbed with murine rickettsiae (Table II) fixed complement with unheated antigens (soluble antigen and also washed rickettsiae) although it failed to do so with the same antigens after they had been heated at 65° C. The unabsorbed serum contained an antibody that fixed complement with the heated antigens.

TABLE II

Effect of heat on the complement-fixing activity of the specific antigens of epidemic and murine rickettsiae

| Test antigen | Date of testing | Treatment | Complement-fixing titre of Breinl convalescent serum | |
|---|---|---|---|---|
| | | | Not treated* | Absorbed with murine rickettsiae |
| Breinl soluble antigen | 18/8/43 | Untreated | 1 in 1600 | 1 in 800 |
| | | Heated at 65° C. | 1 in 400 | <1 in 100 |
| Breinl rickettsiae | | Untreated | 1 in 800 | 1 in 400 |
| | | Heated at 65° C. | 1 in 200 | <1 in 100 |
| Murine soluble antigen | | Untreated | 1 in 400 | <1 in 100 |

* *This serum contained, therefore, antibodies removed by the murine rickettsiae.*

It may, therefore, be concluded that the murine suspension that was used for absorption removed antibody that reacted with an antigen common to the Breinl and murine preparations, and that this common complement-fixing antigen was stable at 65° C. The tests with the absorbed serum, in which, presumably, only the specific antibodies remained, show that the specific complement-fixing antigen is labile at 65° C.

Table III shows the results obtained when a pooled Breinl convalescent serum was absorbed with heated Breinl rickettsiae. It will be noted that rickettsiae heated at 56° C. retained their capacity to absorb antibodies to heated and unheated soluble antigen but that rickettsiae heated at 65° C. failed to absorb antibody to the heat labile antigen.

## II. The Specificity and Heat Lability of the Toxic Antigens of Epidemic and Murine Strains of Rickettsiae

When concentrated suspensions of infective typhus rickettsiae are inoculated intraperitoneally or intravenously into white mice, death occurs in one to eight hours (2, pp. 25–27; 9) Small doses of vaccine protect mice against this

TABLE III

ABSORPTION OF BREINL CONVALESCENT SERUM WITH UNHEATED AND HEATED BREINL RICKETTSIAE

| Treatment of serum | Date of testing | Titre of serum for Breinl soluble antigen treated at the following temperatures | | |
|---|---|---|---|---|
| | | Not heated | 56° C. | 65° C. |
| Untreated | 12/8/43 | 1 in 800 | 1 in 800 | — |
| Absorbed with unheated rickettsiae | | <1 in 50 | <1 in 50 | — |
| Absorbed with rickettsiae heated at 56° C. | | <1 in 50 | <1 in 50 | — |
| Untreated | 24/8/43 | 1 in 800 | — | 1 in 400 |
| Absorbed with unheated rickettsiae | | <1 in 50 | — | <1 in 50 |
| Absorbed with rickettsiae heated at 65° C. | | 1 : 800 | — | <1 in 50 |

— *Not tested.*

toxic effect. This protection has been found to be highly specific when small doses of vaccine are used, although some cross protection is evident when a considerable multiple of the minimal immunizing dose is used (Memo. No. 4 (4) ). A number of experiments have recently been undertaken to obtain further information concerning the specificity and heat stability of the antigens that stimulate immunity to the toxic factor of rickettsiae. Since the results have been consistent, only a few will be described.

The vaccines used were either samples of production lots of vaccine submitted for the routine mouse antigenicity test, or specially prepared small experimental lots. The technique of testing the potency of vaccines has been described in Memorandum No. 6, Part V (6) and need not be detailed here.

Table IV summarizes a comparison of the antigenicity of an experimental vaccine before and after heating at 50° C. and 56° C. for 45 min. The mice received a single dose of 0.5 cc. of diluted vaccine intraperitoneally. The vaccine was so diluted that 0.5 cc. contained 0.01 cc. of vaccine at standard concentration. Three weeks after vaccination, the mice were tested for immunity by intraperitoneal injection of a toxic preparation of rickettsiae.

It would appear from the results of a number of tests that 56° C. is the critical temperature at which inactivation of the specific immunizing antigen becomes apparent. Other tests have shown that vaccine may be kept at 42° C. for several weeks without a demonstrable loss of antigenicity.

Table V shows the results of cross protection tests with the Breinl and Madrid epidemic strains and the Castaneda murine strain. Vaccines heated at 56° C. were compared with untreated vaccine. Doses of vaccine ranging

## TABLE IV

THE EFFECT OF HEAT ON THE IMMUNIZING VALUE OF TYPHUS VACCINE FOR MICE

| Vaccine | Treatment of vaccine | No. of vaccinated mice surviving test with the following doses of yolk sac culture of Breinl strain | | |
|---|---|---|---|---|
| | | $\frac{1}{100}$th | $\frac{1}{200}$th | $\frac{1}{400}$th |
| 56 *ES* 7/4 | Not heated | 8/8** | 8/8 | — |
| 0.01 cc. at | 50° C. for 45 min. | 7/8 | 8/8 | — |
| *S.C.** | 56° C. for 45 min. | 1/8 | 3/8 | — |
| Controls | No vaccine | 0/8 | 3/8 | 5/8 |

* *S.C. = standard concentration.*
** *Denominator = no. of mice tested.*
*Numerator = no. of mice surviving.*

## TABLE V

CROSS PROTECTION TESTS IN MICE WITH EPIDEMIC AND MURINE VACCINES

| Vaccine | Treatment of vaccine | Dose of vaccine at *S.C.* in cc. | Number of mice surviving when tested with following toxic preparations | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Breinl | | | Madrid | | | Murine | | |
| | | | 1/150 | 1/300 | 1/600 | 1/100 | 1/200 | 1/400 | 1/300 | 1/600 | 1/1200 |
| Breinl lot | Not | 0.3 | 4/4 | | | 4/4 | | | 1/4 | | |
| No. 94 | heated | 0.1 | 4/4 | | | 4/4 | | | 4/4 | | |
| | | 0.03 | 4/4 | | | 3/4 | | | 3/4 | | |
| | | 0.01 | 4/4 | | | 3/4 | | | 1/4 | | |
| | | 0.003 | 3/4 | | | 3/4 | | | 2/4 | | |
| | 56° C. for | 0.3 | 2/4 | | | 4/4 | | | 3/4 | | |
| | 45 min. | 0.1 | 4/4 | | | 3/4 | | | 3/4 | | |
| | | 0.03 | 3/4 | | | 4/4 | | | 0/4 | | |
| | | 0.01 | 2/4 | | | 1/4 | | | 1/4 | | |
| | | 0.003 | 0/4 | | | 0/4 | | | 2/4 | | |
| Madrid lot | Not | 0.3 | 4/4 | | | 4/4 | | | 2/4 | | |
| No. 78 | heated | 0.1 | 4/4 | | | 4/4 | | | 1/4 | | |
| | | 0.03 | 4/4 | | | 4/4 | | | 2/4 | | |
| | | 0.01 | 4/4 | | | 3/4 | | | 1/4 | | |
| | | 0.003 | 2/4 | | | 4/4 | | | 1/4 | | |
| | 56° C. for | 0.3 | 1/4 | | | 0/4 | | | 0/4 | | |
| | 45 min. | 0.1 | 0/4 | | | 0/4 | | | 2/4 | | |
| | | 0.03 | 2/4 | | | 0/4 | | | 0/4 | | |
| | | 0.01 | 0/4 | | | 0/4 | | | 1/4 | | |
| | | 0.003 | 0/4 | | | 0/4 | | | 2/4 | | |
| Murine lot | Not | 0.3 | 3/4 | | | 3/4 | | | 4/4 | | |
| No. 92 | heated | 0.1 | 3/4 | | | 2/4 | | | 4/4 | | |
| | | 0.03 | 2/4 | | | 3/4 | | | 4/4 | | |
| | | 0.01 | 0/4 | | | 1/4 | | | 4/4 | | |
| | | 0.003 | 1/4 | | | 0/4 | | | 4/4 | | |
| | 56° C. for | 0.3 | 2/4 | | | 1/4 | | | 0/4 | | |
| | 45 min. | 0.1 | 0/4 | | | 0/4 | | | 0/4 | | |
| | | 0.03 | 0/4 | | | 0/4 | | | 0/4 | | |
| | | 0.01 | 0/4 | | | 1/4 | | | 0/4 | | |
| | | 0.003 | 0/4 | | | 0/4 | | | 0/4 | | |
| Controls | | None | 0/8 | 2/8 | 5/8 | 0/8 | 2/8 | 6/8 | 0/8 | 4/8 | 5/8 |

*Note: Breinl challenge—26/7/43; Madrid challenge—3/8/43; murine challenge—28/7/43.*

from 0.003 cc. to 0.3 cc. at standard concentration were used in order to obtain quantitative data on the heat lability of the toxic antigen and on the relationship of the epidemic and murine antigens.

The results obtained with mice inoculated with unheated vaccine and tested with toxic preparations of the homologous strain of rickettsiae indicate a considerable degree of specific protection*. The cross tests with the Breinl and Madrid strains do not reveal any difference between these two epidemic types. In contrast to this specific protection, there is evidence of an irregular cross protection between the epidemic and murine strains. When vaccines in this experiment were given in doses 30 to 100 times greater than their minimal immunizing dose for the homologous type they protected only 40 to 75% of mice against the heterologous type.

The inactivation of the immunizing antigen at 56° C. is well shown in the case of the Madrid and murine vaccines. The Breinl vaccine on this occasion was not completely inactivated at 56° C., although the results show that significant reduction occurred. In this connection, the possible influence of traces of formalin on stability at the critical temperature should be borne in mind.

## III. The Differentiation of Specific and Other Neutralizing Antibodies to Epidemic and Murine Strains of Typhus Rickettsiae by Means of the Giroud Rabbit Skin Reaction

Giroud (10) found that typhus immune serum neutralized the capacity of preparations of rickettsiae to produce a cutaneous reaction in rabbits. This cutaneous serum protection test has not given entirely satisfactory results in the hands of some investigators, but the possible advantages of a reliable skin test for rickettsial neutralizing antibodies prompted a further study. Encouraging results have been obtained by the introduction of certain modifications of the method. These modifications are based on the following observations.

There is a qualitative difference between the lesions produced (*a*) by a suspension of infective rickettsiae that has had its activity reduced by simple dilution and (*b*) the same suspension that has had its activity reduced as a result of exposure to immune serum. This difference is primarily one of ratio of the diameter to the thickness of the lesions. A partially neutralized mixture produces a lesion that is elevated, bright to dusky red in colour, with a clearly defined border. The diameter of the lesion is less than that of the lesion produced by the non-neutralized control mixture. Exactly similar lesions are not produced by higher dilutions of the rickettsial suspension in protective fluid; the redness and thickness of the lesion varies with the dilution of the material and with higher dilutions the border is poorly defined. If a

*Addendum, 2nd October, 1943.

*Since this paper was prepared, cross immunity tests have been carried out in which vaccinated mice were tested by intravenous injection of toxic preparations. The results confirm the specificity of immunity to the toxic factors of epidemic and murine strains and also the heat lability of the toxic antigen.*

sufficient dose of infective virus, i.e., one that produces a well marked lesion, is used to test for neutralizing antibody, there is no difficulty in making accurate comparative measurements of the lesions produced by the control and immune serum mixtures.

A study of the quantitative relationships between the concentrations of virus and serum has shown that neutralization follows the percentage law first described by Andrewes and Elford in the case of bacteriophage neutralization and later shown to be operative in the neutralization of viruses (3). The appearance of complete neutralization can be obtained only with high titre sera and a relatively weak concentration of virus. Consequently, any method that attempts to titrate sera to a null end-point sacrifices sensitivity and consistency of results. More satisfactory results are obtained when the estimation of neutralizing antibody is based on the relative degree of reduction of the size of the lesion. Such estimation is possible because there is a relationship between the concentration of antibody and the dimensions of the lesion. This relationship will be explained after a brief description has been given of the procedure followed in carrying out the neutralization test.

Three fourfold dilutions are made of the serum to be tested. A standard concentration of a yolk sac culture of rickettsiae, which is used also as a reference control, is added to each dilution and the mixtures are shaken at intervals during a period of two hours at room temperature. Each mixture is then diluted 1 in 30, and 0.2 cc. volumes of the dilutions are injected intradermally. The maximum and minimum diameters of the lesions are measured at their height on the third or fourth day. In addition, the thickness of the lesion is estimated on a scale of 0, ½, 2, 3, or 4. The thickness of the reference control lesions should have a value of 3 or 4.

Let us suppose then that $A$ represents the cube root of the product of the three dimensions of the unneutralized control, and that $X_1$, $X_2$, and $X_3$, respectively, represent the cube roots of the products of the dimensions of each of the three lesions representing the three dilutions of immune serum.

If $X_1$, $X_2$, and $X_3$ are plotted as abscissa on a uniform scale against the corresponding dilution of serum as ordinate on a logarithmic scale, the three points will lie approximately on a straight line. The slope of this line is a constant for all sera tested together on one animal. This line is extrapolated to intersect a line drawn parallel with the ordinate from a point on the abscissa representing $A$, the size of the control lesion*. The point of intersection of these lines indicates the titre of the serum. A reference control serum is used in each group of tests because of variations in the skin sensitivity of rabbits. This use of a reference serum permits correction of the calculated titre of sera with the result that the titre can be determined with an accuracy that is within the limits of a twofold dilution.

Observations on many guinea-pig and human sera have shown that epidemic immune sera neutralize murine virus to a significant extent, and conversely,

* *The size of the control lesion is permitted to vary from 14 × 14 × 3 mm. to 17 × 17 × 4 mm. in different tests in different rabbits.*

that murine sera neutralize epidemic virus. The senior author had found that the typical rabbit skin reaction, which reaches its maximum in three to four days depends on the presence of infective rickettsiae and that high concentrations of killed rickettsiae are required to produce a transient skin reaction. This latter reaction reaches its height in 24 to 36 hr. and fades rapidly. The same author was also able to demonstrate that immune serum prevented this reaction. These observations suggested that neutralizing antibody inhibition tests with non-infective rickettsiae might indicate whether epidemic and murine immune sera contained two distinct neutralizing antibodies.

These inhibition tests were carried out with the following materials.

*Sera.*—These were obtained from guinea-pigs that had been injected with yolk sac cultures of the Breinl epidemic or the Castaneda murine strains. The bleedings were made 21 days after the onset of fever.

*Inhibiting Suspensions of Rickettsiae.*—The rickettsiae were separated by centrifugation from typhus vaccine processed as described in Memorandum No. 5 (5), suspended in saline and then purified further with ethyl ether. The concentration of rickettsiae in these repurified stocks was determined photometrically.

*Infective Cultures of Rickettsiae Used to Test for Neutralizing Antibody.*—These were yolk sac cultures, prepared and preserved in the same way as the toxic material used in the mouse test (6). The preparations were subjected to a preliminary titration to determine their optimum dilution for the estimation of neutralizing antibody. The Madrid No. IV strain was used in preference to the Breinl to test for epidemic type antibody since it produces a more marked and uniform reaction.

Two sets of inhibition tests were carried out, one with Breinl convalescent serum, the other with murine convalescent serum. In both sets of tests the following inhibiting suspensions were used.

(*a*) Breinl, not heated.
(*b*) Breinl, heated at 56° C. for 45 min.
(*c*) Murine, not heated.
(*d*) Murine, heated at 56° C. for 45 min.

Each suspension was mixed with 0.125 cc. of serum and saline was added to bring the volume to 1 cc. thus giving a 1 in 8 dilution of serum. The amounts of suspension that were used were such as to contain approximately 0.125 mgm. to 0.5 mgm. of rickettsiae (dry weight estimated from photometric data). After addition of the inhibiting suspension, the mixtures were shaken at intervals and allowed to stand overnight at 4° C. Thereafter, each mixture was divided into two parts. One of these was added to an infective yolk sac preparation of the Madrid strain and the other was added to a murine preparation. After a period of two hours at room temperature, during which the mixtures were shaken frequently, each mixture was diluted 1 in 30 and 0.2

cc. was injected intradermally. The resultant lesions were measured on the third and fourth days.

The results of the tests are presented in a simplified form in Figs. 3 and 4. The length of each column is the reciprocal of the size of the lesion produced.

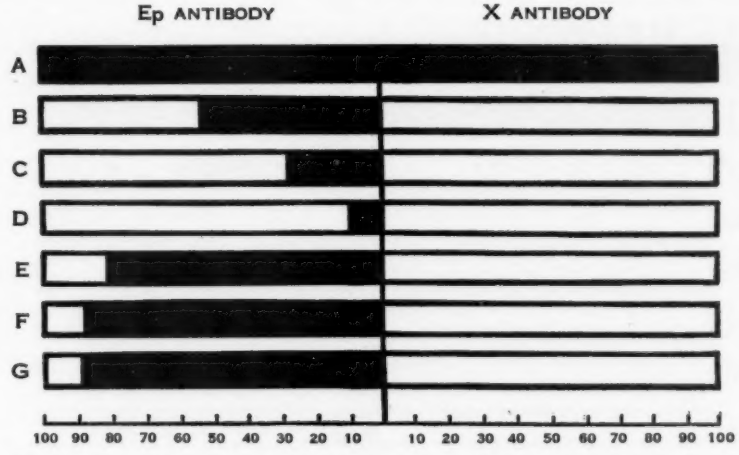


FIG. 3. *Neutralizing antibody inhibition tests; Breinl convalescent guinea-pig serum. Percentage of free neutralizing antibody remaining after addition of the inhibiting suspension is indicated in black.*

*Ep—Antibody neutralizing the Madrid No. 4 strain.*

*X—Antibody neutralizing the murine strain.*

*A—Untreated serum control containing 225 units of Ep antibody and 55 units of X antibody.*

*B—Serum inhibited with 0.125 mgm. of Breinl suspension.*

*C—Serum inhibited with 0.25 mgm. of Breinl suspension.*

*D—Serum inhibited with 0.5 mgm. of Breinl suspension.*

*E—Serum inhibited with 0.125 mgm., 0.25 mgm., or 0.5 mgm. of Breinl suspension heated at 56° C.*

*F—Serum inhibited with 0.5 mgm. of murine suspension.*

*G—Serum inhibited with 0.5 mgm. of murine suspension, heated at 56° C.*

*Residual X antibody less than 10% in E and less than 5% in B, C, D, F, and G.*

Since there is a quantitative relationship between the amount of neutralizing antibody and the reduction in size of the skin lesion, these reciprocals may, for the present purpose, be taken as equivalent to the percentage of antibody that was not inhibited. The titres of the uninhibited serum controls were as follows:

Breinl serum: 225 neutralizing units for Madrid virus, and 55 neutralizing units for murine virus.

Murine serum: 230 neutralizing units for murine virus, and 45 neutralizing units for Madrid virus.

The results are analogous to those reported for complement fixing antibodies in Section II of this paper. Inhibition with the heterologous type failed to

remove neutralizing antibody to the homologous type. Thus, it would appear that there are two neutralizing antibodies that participate in the Giroud cutaneous serum protection test, one representing a specific antigen, the other an antigen shared by epidemic and murine types. The inhibition tests with heated suspensions of rickettsiae show that the specific antigens are labile at 56° C. (Figs. 3 and 4).

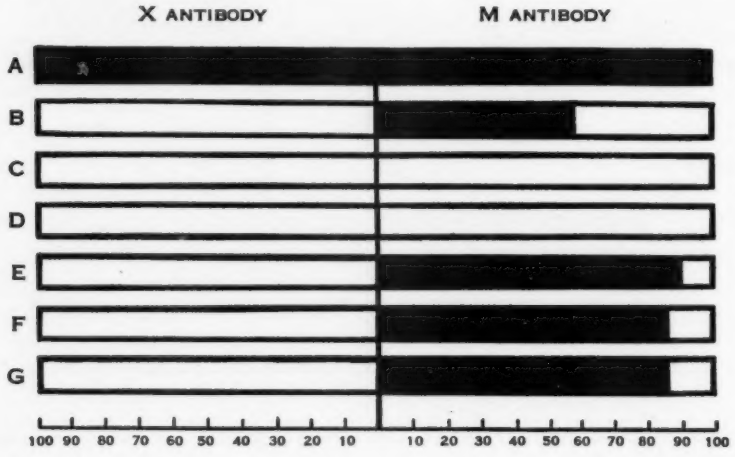


FIG. 4. *Neutralizing antibody inhibition tests; murine convalescent guinea-pig serum. Percentage of free neutralizing antibody remaining after addition of the inhibiting suspension is indicated in black.*

*M—Antibody neutralizing the murine strain.*

*X—Antibody neutralizing the Breinl strain.*

*A—Untreated serum control containing 230 units of M antibody and 45 units of X antibody.*

*B—Serum inhibited with 0.125 mgm. of murine suspension.*

*C—Serum inhibited with 0.25 mgm. of murine suspension.*

*D—Serum inhibited with 0.5 mgm. of murine suspension.*

*E—Serum inhibited with 0.125 mgm., 0.25 mgm., or 0.5 mgm. of murine suspension heated at 56° C.*

*F—Serum inhibited with 0.5 mgm. of Breinl suspension.*

*G—Serum inhibited with 0.5 mgm. of murine suspension heated at 56° C.*

*Residual X antibody less than 5% in B, C, D, E, F, and G.*

## IV. The Neutralizing Antibody Response of Guinea-pigs Inoculated Intraperitoneally with Typhus Vaccine Heated at 60° C.

The results of some preliminary comparative tests of untreated and heated vaccine are of interest since they suggest a diversity of rickettsial antibodies and the need of further study to elucidate the relative importance of these antibodies in immunity.

The vaccine used in these tests was prepared from the yolk sacs of eggs inoculated on the sixth day of development with the equivalent of one-millionth of a yolk sac of Breinl culture. Eleven yolk sacs were collected

from the 10th to 12th day after inoculation and showed a particularly heavy infection. They were processed as described in Memorandum No. 5 (5). The vaccine thus obtained (56 *ES/D* 8/2(2)) showed the high dilution factor of 1 in 11.2. At this dilution, the vaccine represented less than a 1% suspension of yolk sac by weight. The preceding transfers of the Breinl strain in this series of egg passages had involved mouse blood and embryo blood transfers, and mouse antigenicity tests were therefore undertaken to determine whether any demonstrable antigenic change or degradation had occurred. The results of these antigenicity tests, carried out with 0.01 cc. of the vaccine diluted 1 in 11.2 to reduce it to standard concentration, provided further evidence that the immunizing value of vaccine prepared by the methods described in Memorandum No. 5 (5) is primarily related to the concentration of rickettsiae present.

At a later date, in connection with investigation of the heat lability of the mouse immunizing antigen, mice and guinea-pigs were vaccinated with this vaccine (56 *ES/D* 8 2(2)). The mice received the usual dose equivalent to 0.01 cc. at standard concentration. The guinea-pigs received 0.5 cc. of vaccine in terms of a 10% yolk sac suspension. The vaccines were heated in a water-bath regulated within 0.2° C. and were used on the same day. The intraperitoneal route of inoculation was used for all animals. Three weeks after vaccination, the mice were tested for active immunity and the guinea-pigs were bled out.

TABLE VI

VACCINE 56 *ES/D* 8/2(2)

MOUSE ANTIGENICITY TEST

| Treatment of vaccine | Dose of vaccine at standard concentration | No. of mice surviving test with following doses of Breinl yolk sac | | |
|---|---|---|---|---|
| | | $\frac{1}{120}$ | $\frac{1}{240}$ | $\frac{1}{480}$ |
| None | 0.01 cc. | 8/8 | 8/8 | — |
| Heated at 56° C. for 45 min. | 0.01 cc. | 2/8 | 8/8 | — |
| | 0.03 cc. | 3/8 | 8/8 | — |
| | 0.1 cc. | 8/8 | 7/8 | — |
| | 0.3 cc. | 8/8 | 8/8 | — |
| Controls | No vaccine | 0/8 | 1/8 | 4/8 |

Table VI indicates that a temperature of 56° C. for 45 min. reduced the antigenicity of the vaccine for mice by approximately 90%. Table VII*b* shows that heating at 60° C. for 45 min., which markedly reduced the mouse antigenicity (Table VII*a*), did not appreciably affect the ability of the vaccine to stimulate complement-fixing antibodies in tests that did not differentiate antibodies for the heat labile and heat stable antigens (Table VII*b*). In fact, the mean response to the heated vaccine was slightly greater than the

TABLE VII*a*

VACCINE 56 *ES/D* 8/2(2). Heated 7/5/43

MOUSE ANTIGENICITY TEST

| Treatment of vaccine | Dose of vaccine at standard concentration | No. of mice surviving test with following doses of Breinl yolk sac | | |
|---|---|---|---|---|
| | | $\frac{1}{250}$ | $\frac{1}{500}$ | $\frac{1}{1000}$ |
| None | 0.01 cc. | 6/8 | 8/8 | — |
| Heated at 60° C. for 45 min. | 0.01 cc. | 1/8 | 4/8 | — |
| Controls | No vaccine | 0/8 | 5/8 | 8/8 |

TABLE VII*b*

VACCINE 56 *ES/D* 8/2(2). HEATED 7/5/43

COMPLEMENT-FIXING ANTIBODY RESPONSE OF GUINEA-PIGS INOCULATED INTRAPERITONEALLY WITH 5 CC. AT *S.C.* I.E. 0.5 CC. OF THE EQUIVALENT OF A 10% YOLK SAC SUSPENSION

| Treatment of vaccine | Guinea-pig number | Complement-fixing titre of serum with following soluble antigens | | |
|---|---|---|---|---|
| | | Breinl | Madrid | Murine |
| None | 2089 | Not tested | — | — |
| | 2090 | Partial 1 in 100 | Partial 1 in 100 | Neg. 1 in 100 |
| | 2091 | 1 in 400 | 1 in 400 | Neg. 1 in 100 |
| | 2092 | Neg. 1 in 50 | Neg. 1 in 50 | Neg. 1 in 50 |
| | 2093 | Not tested | — | — |
| | 2094 | 1 in 800 | 1 in 800 | 1 in 200 |
| Heated at 60° C. for 45 min. | 2095 | 1 in 400 | 1 in 400 | 1 in 200 |
| | 2096 | 1 in 400 | 1 in 400 | Partial 1 in 100 |
| | 2097 | 1 in 1600 | 1 in 1600 | 1 in 800 |
| | 2098 | Neg. 1 in 100 | Neg. 1 in 100 | Neg. 1 in 100 |
| | 2099 | Neg. 1 in 100 | Neg. 1 in 100 | Neg. 1 in 100 |
| | 2100 | 1 in 100 | 1 in 100 | Partial 1 in 100 |

mean response to the untreated vaccine. The guinea-pig sera were pooled and tested for neutralizing antibody, by the modified Giroud test described in Section III, and also by the mouse method described by Henderson and Topping (12, pp. 41–56). The results of these tests are given in Table VII*c*.

Another set of tests was carried out on the same vaccine heated at 60° C. on another occasion (Tables VIII*a* and VIII*b*). Again, a lack of correlation between complement-fixing antibody and neutralizing antibody response was evident. As in the previous experiment, the heated vaccine stimulated neutralizing antibodies as shown by the Giroud test with the Madrid epidemic strain. Two pairs of sera, representing unheated and heated vaccines,

TABLE VII*c*

VACCINE 56 *ES/D* 8/2/(2). HEATED 7/5/43

NEUTRALIZING ANTIBODY RESPONSE OF GUINEA-PIGS INOCULATED INTRAPERITONEALLY (SEE TABLE I)

MOUSE TEST 26/8/43

| Treatment of vaccine | Pool guinea-pig sera | Neutralizing units by Giroud test with Madrid strain | Serum–virus mixture | | No. of mice surviving intravenous inoculation of serum–virus mixtures | |
|---|---|---|---|---|---|---|
| | | | Dilution of serum | Fraction of Breinl yolk sac | For 6 hr. | For 24 hr. |
| None | 2090 | 250 | 1 in 32 | 1/320 | 6/6 | 6/6 |
| | 2091 | | 1 in 64 | 1/320 | 6/6 | 6/6 |
| | 2092 | | 1 in 128 | 1/320 | 6/6 | 5/6 |
| | 2094 | | 1 in 256 | 1/320 | 0/6 | 0/6 |
| | | | 1 in 512 | 1/320 | 0/6 | 0/6 |
| Heated at 60° C. for 45 min. | 2095 | 60 | 1 in 16 | 1/320 | 6/6 | 6/6 |
| | 2096 | | 1 in 32 | 1/320 | 0/6 | 0/6 |
| | 2097 | | 1 in 64 | 1/320 | 0/6 | 0/6 |
| | 2098 | | 1 in 128 | 1/320 | 0/6 | 0/6 |
| | 2099 | | 1 in 512 | 1/320 | 1/6 | 1/6 |
| | 2100 | | | | | |
| Controls | Normal guinea-pig serum | | 1 in 16 | 1/320 | 0/6 | 0/6 |
| | | | 1 in 16 | 1/640 | 1/6 | 1/6 |
| | | | 1 in 16 | 1/1280 | 6/6 | 6/6 |

TABLE VIII*a*

VACCINE 56 *ES/D* 8/2(2). HEATED 3/5/43

COMPLEMENT-FIXING AND NEUTRALIZING ANTIBODY RESPONSE OF GUINEA-PIGS INOCULATED INTRAPERITONEALLY WITH 5 CC. AT *S.C.*, I.E. 0.5 CC. OF THE EQUIVALENT OF A 10% YOLK SAC SUSPENSION

| Treatment of vaccine | Guinea-pig No. | Neutralizing units by Giroud test with Madrid strain | Complement-fixing titre of serum with following soluble antigens | | |
|---|---|---|---|---|---|
| | | | Breinl | Madrid | Murine |
| None | 2070 | Not tested | 1 in 400 | 1 in 400 | Neg. 1 in 100 |
| | 2071 | Neg. | Neg. 1 in 125 | Neg. 1 in 125 | Neg. 1 in 125 |
| | 2072 | 42 | 1 in 200 | 1 in 200 | 1 in 100 |
| | 2074 | 110 | 1 in 200 | 1 in 100 | Neg. 1 in 125 |
| | 2076 | 85 | 1 in 800 | 1 in 400 | Neg. 1 in 100 |
| | 2077 | 8 | 1 in 200 | 1 in 400 | Neg. 1 in 100 |
| Heated at 60° C. for 45 min. | 2078 | Not tested | — | — | — |
| | 2079 | 160 | 1 in 200 | 1 in 200 | 1 in 100 |
| | 2081 | Not tested | 1 in 200 | 1 in 200 | 1 in 100 |
| | 2082 | 25 | 1 in 400 | 1 in 400 | 1 in 200 |
| | 2083 | 17 | 1 in 100 | 1 in 200 | Neg. 1 in 100 |
| | 2084 | 85 | 1 in 400 | 1 in 400 | 1 in 400 |

TABLE VIII*b*

VACCINE 56 *ES/D* 8/2(2). HEATED 3/5/43

NEUTRALIZING ANTIBODY RESPONSE OF GUINEA-PIGS INOCULATED INTRAPERITONEALLY WITH 5 CC. AT *S.C.* (SEE TABLE II)

| Treatment of vaccine | Pool of guinea-pig sera | Neutralizing units, by Giroud test with Madrid strain | Serum virus mixture | | No. of mice surviving intravenous inoculation of serum virus mixture | |
|---|---|---|---|---|---|---|
| | | | Dilution of serum | Fraction of Breinl yolk sac | For 6 hr. | For 24 hr. |
| None | 2074 | 110 | 1 in 32 | 1/320 | 6/6 | 6/6 |
| | 2076 | 85 | 1 in 64 | 1/320 | 6/6 | 6/6 |
| | | | 1 in 128 | 1/320 | 6/6 | 6/6 |
| | | | 1 in 256 | 1/320 | 1/6 | 1/6 |
| | | | 1 in 512 | 1/320 | 1/6 | 1/6 |
| Heated at 60° C. for 45 min. | 2079 | 160 | 1 in 8 | 1/320 | 6/6 | 6/6 |
| | 2084 | 85 | 1 in 16 | 1/320 | 6/6 | 6/6 |
| | | | 1 in 32 | 1/320 | 6/6 | 6/6 |
| | | | 1 in 64 | 1/320 | 0/6 | 0/6 |
| | | | 1 in 128 | 1/320 | 0/6 | 0/6 |
| Controls | Normal serum | — | 1 in 8 | 1/320 | 0/6 | 0/6 |
| | | | 1 in 8 | 1/640 | 0/6 | 0/6 |
| | | | 1 in 8 | 1/1280 | 6/6 | 6/6 |

respectively, were selected on the basis of the results of the Giroud test and pooled for mouse test. The results of the mouse neutralization test are given in Table VIII*b*. Although the sera from the guinea-pigs receiving heated vaccine had a neutralizing titre of 1 in 32, there was no correlation between the neutralizing titres determined by the rabbit skin test and the mouse. This, however, means very little since other observations (Section III) demonstrate the existence of two neutralizing antibodies participating in the the Giroud serum protection test and the consequent necessity of resorting to inhibition test to determine the titre of specific neutralizing antibody.

## Discussion

The observations that have been described indicate that epidemic and murine typhus rickettsiae have the antigenic structures shown diagrammatically in Fig. 5. The evidence in support of this scheme may be summarized as follows, according to the kind of test used to differentiate the antibodies developed in response to the specific and common antigens.

### *Agglutination Tests*

Earlier tests by the senior author in which use was made of agglutinating sera produced in the rabbit showed that absorption of Breinl sera with murine rickettsiae, and vice versa, resulted in removal of antibody responsible for cross agglutination reactions but that specific antibody remained. The antigen reacting with the specific antibody was found to be labile at 56° C.

A recent report (1) describes more extensive observations on a variety of strains, and the writers of this report conclude that both murine and epidemic rickettsiae possess a specific "species" antigen and share another antigen common to strains of the *OX*19 group.

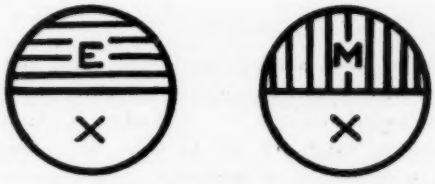


FIG. 5. *Antigenic structure of epidemic and murine typhus rickettsiae.*
*E—heat labile specific epidemic antigen.*
*M—heat labile murine specific antigen.*
*X—heat stable common antigen.*

*Complement Fixation Tests*

Absorption of convalescent guinea-pig serum with typhus rickettsiae of the type causing the infection removes antibodies to the homologous and heterologous types with equal facility. The amount of antibody removed is proportional to the absorbing dose. Absorption of the same serum with typhus rickettsiae of the heterologous type gives an entirely different result. In this instance, the antibody that reacts with the heterologous type is removed quantitatively in proportion to the absorbing dose, but antibody that reacts specifically with the homologous type is not removed. These effects are shown in Fig. 1. It is clearly evident that the Breinl serum contains antibodies that are not absorbed by the murine suspension. Similarly, the murine serum contains antibodies that are not absorbed by the Breinl suspension. There seems to be no alternative to the conclusion that epidemic and murine strains share a common complement-fixing antigen stable at 65° C. and that each possesses, in addition, its own specific heat labile antigen. The murine strain has no demonstrable trace of specific epidemic antigen and the converse applies to the epidemic strains.

*The Giroud Test for Neutralizing Antibodies*

As shown in Section III (Figs. 3 and 4) it is possible to demonstrate inhibition of neutralizing antibodies. The inhibition test developed for the differentiation of antibodies is the analogue of the absorption test as applied to agglutinating and complement-fixing antibodies. It differs from the absorption test in that it is unnecessary to remove inhibiting antigens from the serum.

Here again, the same antigenic pattern emerges (see Figs. 3 and 4) as in the case of the complement-fixing antigens. The antibodies measured in this case, however, are neutralizing antibodies. It follows, therefore, that the shared, or common, X antigen stimulates the development of antibodies that function as neutralizing antibodies when the Giroud rabbit cutaneous reaction

is employed to measure the percentage of rickettsiae surviving contact with the serum. Thus, the neutralizing titre of a whole serum, as measured by the rabbit skin test, merely indicates the sum or combined effect of two neutralizing antibodies that may be present in varying proportions in different sera.

*The Mouse Test for Neutralizing Antibodies*

Although no definite proof is available, it seems reasonable to assume that active immunity of mice to intraperitoneal inoculation of toxic preparations is predominantly humoral, because neutralization of the toxic factor *in vitro* can be so clearly demonstrated (12, pp. 41–56). Since the heterologous type of vaccine confers no regular cross immunity even when large doses are used in the mouse antigenicity test (Table V), it may be inferred that an antibody developed to the specific antigen is essential to complete immunity to the toxic factor. This does not mean, of course, that antibody to the common X antigen does not play an important role in active immunity. Perhaps both antigens are equally important.

Hamilton (11) has investigated the specificity of epidemic and murine rickettsial toxins and has concluded that the antibodies neutralizing these toxins are immunologically distinct. In the absorption tests reported by Hamilton, rickettsiae of heterologous type failed to remove the toxin-neutralizing antibody to the homologous type. He also noted that the neutralizing antibody was more readily absorbed by rickettsiae than were the complement-fixing antibodies.

It has been observed that vaccine heated at 60° C. in order to inactivate the mouse immunizing antigen, retained its ability to stimulate the development of neutralizing antibody in the guinea-pig (Tables VII*c* and VIII*b*). This stresses the need for further investigation of the complex antigenic structure of rickettsiae and the apparent multiplicity of corresponding antibodies. It should be noted that the guinea-pig sera used in the tests presented in Tables VII*b*, VII*c*, VIII*a*, VIII*b* were obtained by intraperitoneal, not subcutaneous, inoculation of heated vaccine. These sera appeared to neutralize the toxic factor for mice less effectively than they neutralized the skin-reacting factor (Tables VII*c*, VIII*b*). It will be necessary to resort to cross absorption tests before this difference in the results of the two neutralization tests can be satisfactorily explained.

Reference should be made to Felix's suggestion of an analogy between the heat labile and heat stable antigens of rickettsiae and the Vi and O antigens of *B. typhosum*. This analogy seems fully justified. In regard to pathogenic rickettsiae in general, Felix has expressed the opinion that further subdivision will be made possible by the study of the heat labile rickettsial antigens. Perhaps the analogy between the heat labile antigens of typhus rickettsiae and the Vi antigen of *B. typhosum* is more complete than Felix has ventured to suggest. The apparent differences between the heat lability of the specific mouse immunizing antigen (56° C.) and the specific complement fixing antigen

(60° C.), and the discrepant results of active immunity and neutralization tests (particularly the Giroud test) recall the "functional deficiency" of the Vi antigen described by Felix and Bhatnagar (8). Here, however, the analogy would appear to end because formalin seems to promote stability of the labile mouse immunizing antigen.

In conclusion, it should be pointed out that the observations reported here do not exclude the possibility that some strains of rickettsiae develop small amounts of the heat labile specific antigen that predominates in the heterologous type. It will be necessary to resort to cross absorption tests with heated rickettsiae and to an antigenic analysis of intermediate strains in order to obtain information about this interesting question. At least it can be said that the immunological reactions of the strains that have been examined are dominated by the two specific heat labile antigens and the heat stable X antigen.

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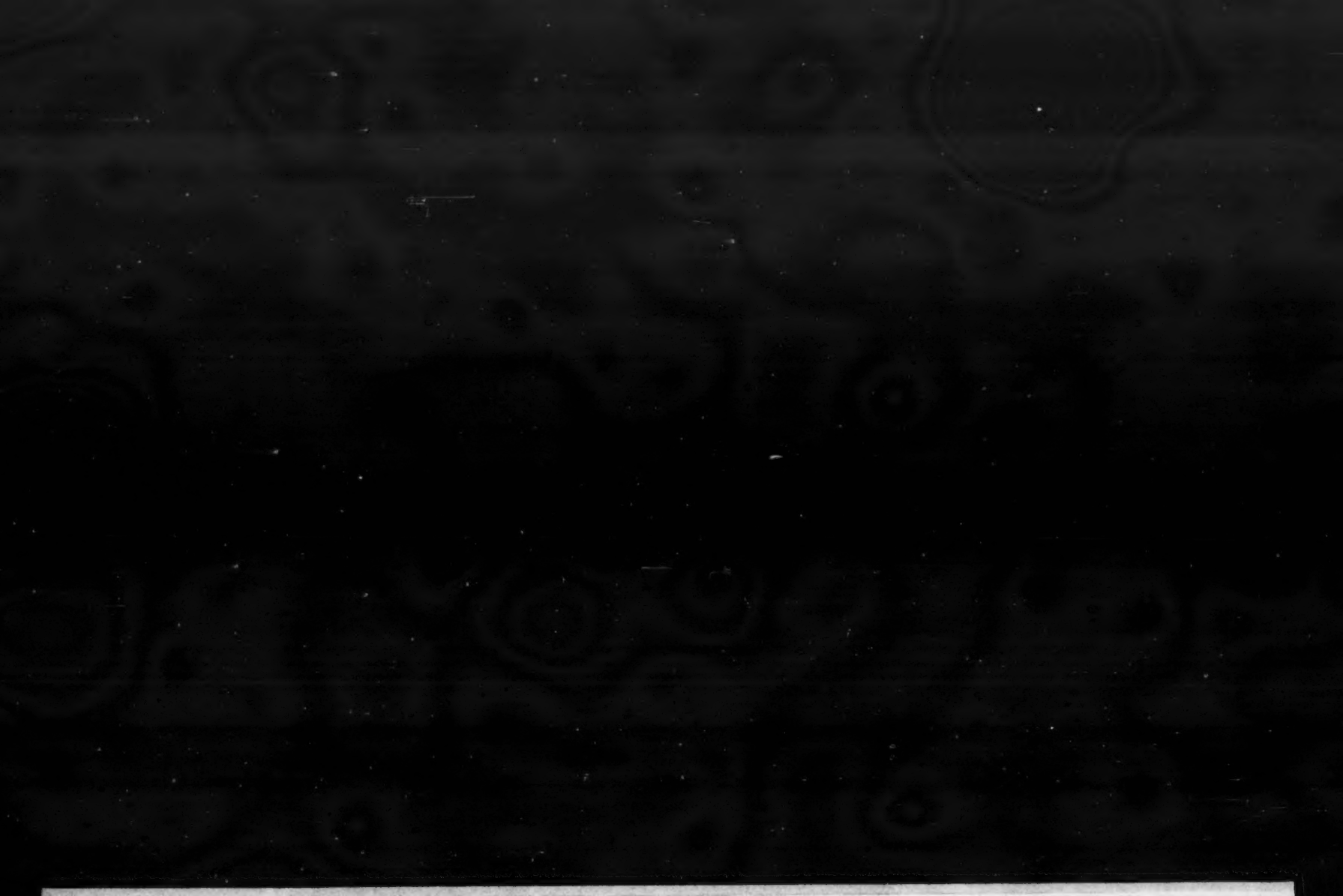
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